STUDIES IN OXIDATIVE PHOSPHORYLATION

A Thesis submitted to the University of Warwick in fulfilment of the degree of Doctor of Philosophy

by

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SUMMARY

Trialkyl tin compounds have been shown to be potent inhibitors of oxidative phosphorylation, oligomycin sensitive ATPase activity and other ATP-dependent reactions of beef heart mitochondria. Unlike oligomycin inhibition of these reactions, trialkyl tin inhibition is reversible by dithiols such as 2,3-dimercaptopropanol. The OS-ATPase activity was found to be 6-10 times more sensitive than oxidative phosphorylation to inhibition by trialkyl tins. This differential sensitivity to trialkyl tin inhibition is discussed in relation to the current theories on energy coupling.

Binding studies with [^{3}H]-DBCT have indicated the presence of two types of binding sites designated high affinity ($k_D \sim 0.3$ μM) and low affinity ($k_D \sim 30$ μM) binding sites. The concentration of the high affinity binding site is ∿ 2.0 nmol/mg protein in submitochondrial particles and saturation of these sites correlated with the inhibition of the oligomycin sensitive ATPase activity. Extraction and isolation experiments have shown that DBCT binds to a small lipophilic, non-protein molecule.

Dihydrolipoic acid has been shown to drive ATP synthesis by acting as a NAD-linked substrate. Fatty acids, oleoyl-phosphate, oleoyl-lipoate and other lipids were found to inhibit succinate driven ATP synthesis and other energy-linked reactions in mitochondria and submitochondrial particles. In addition, a new method for the measurement of nanomoles amount of lipoic acid and lipoamide is reported. The method involves the cyclic reduction of 5,5-dithiobis (2-nitrobenzoic acid) by NADH *via* a system containing lipoamide dehydrogenase and lipoic acid (or lipoamide).

"There are few, if any, certainties in science; we build up our knowledge by testing preconceived models experimentally, thus detecting and discarding the concepts that are false and retaining the concepts that show by their survival that they are factually serviceable because they represent reality as far as it is known."

P. Mitchell, 1977

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ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
AMP-(PNP)	Adenyl imidodiphosphate
ATPase	Adenosine triphosphatase
BAL	2,3-dimercaptopropanol
DBCT	Dibutylchloromethyl tin chloride
DBT	Dibutyl tin dichloride
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DCPIP	2,6-dichlorophenol indophenol
DTT	Dithiothreitol
DCCD	N,N'-dicyclohexylcarbodiimide
EDTA	Ethylenediamine tetra-acetic acid
F_1	Soluble mitochondrial ATPase or coupling factor I
FCCP	Carbonyl cyanide p-trifluoromethoxyphenyl-hydrazone
G-6-P	Glucose-6-phosphate
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
OS-ATPase	Oligomycin-sensitive ATPase
Pi or P	Inorganic phosphate
PCA	Perchloric acid
TTFB	4,5,6,7-tetrachloro-2-(trifluoromethyl) benziimidazole
TCA	Trichloroacetic acid
Tris-Cl	Tris(hydroxymethyl)aminomethane hydrogen chloride

$\Delta\psi$	Membrane potential
1799	Bis-(hexafluoroacetonyl)acetone

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CHAPTER 1

1. INTRODUCTION

1.1 GENERAL INTRODUCTION

Elucidation of the mechanism by which energy liberated in cellular electron transport is conserved and utilised for ATP synthesis, has been a major challenge in biochemistry ever since the occurrence of phosphorylation in the respiring cell was first recognised in the 1930s [see (1, 2, 3) for reviews]. Today, it is well established that the electron transport system associated with the inner membrane of mitochondria, the thylakoid membrane of chloroplasts and the plasma membrane of respiring and photosynthetic prokaryotes are functionally linked to an ATP-synthesising system by a mechanism that is fundamentally similar in all living cells. It is also well established that the energy deriving from the oxidation of reducing equivalents by the respiratory chain is converted to ATP by the ATP-synthase complex. Before discussing the mechanisms involved in this process, the structure and function of the respiratory chain will be briefly outlined [see (6, 7, 8) for more in depth reviews].

1.2 THE RESPIRATORY CHAIN

Current ideas of the component composition and sequence of the electron transport chain are shown in Fig. 1.1 (4). This scheme has emerged from the confluence of several lines of investigation. They include measurement of the redox potential of individual components (6), kinetic determination of the reaction sequence (6, 7), studies on the donor and acceptor specificity of isolated components (6) and the interaction of electron transfer inhibitors with the respiratory chain (6, 8). Chemical fractionation

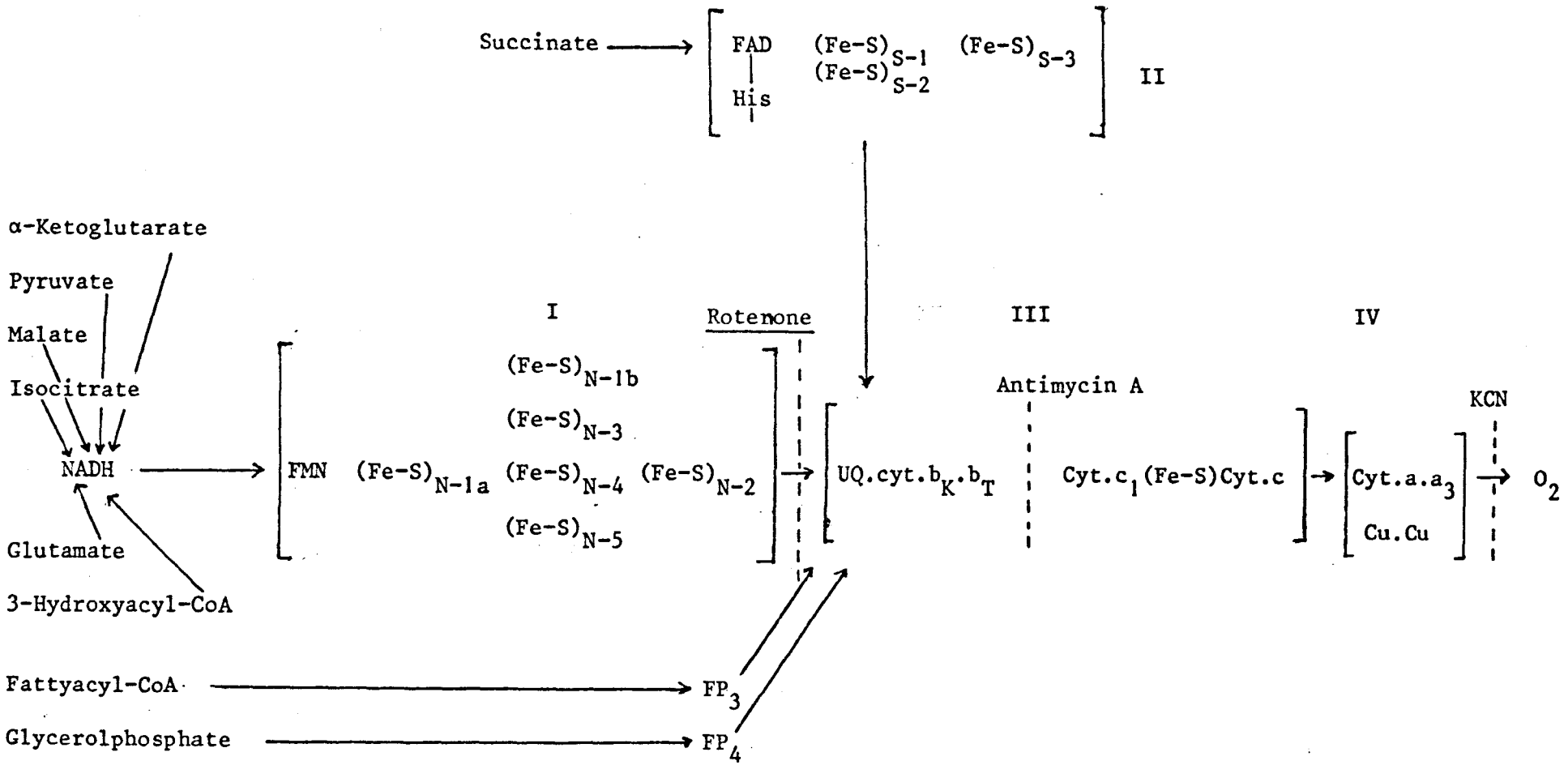
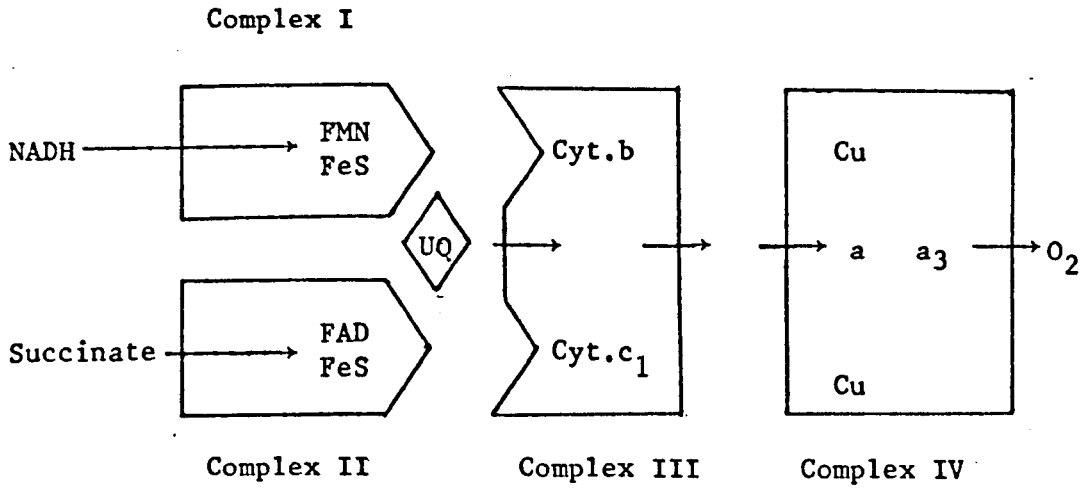


Fig. 1.1 The electron transfer components of the respiratory chain arranged as a continuous sequence from NADH to oxygen. The points of entry of electrons from various substrates are shown, as well as the sites of inhibition of electron transport. The symbol FP designates flavoproteins; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; (Fe-S), iron-sulphur centres where the subscript N or S indicates NADH and succinate. UQ is ubiquinone (coenzyme Q) and Cyt. the cytochromes b_K, b_T, c, c_1, a and a_3, Cu copper. The bracketed portions list the total component in each complex. [Adopted from Ohnishi 1975 (4)].

studies by Hatefi et al. have shown that the respiratory chain can be split into four complexes, designated complex I, II, III and IV according to the classification of D. E. Green (9-11). The complexes catalyse the partial reactions shown in Fig. 1.2 and can be recombined stoichiometrically to give a reconstituted respiratory chain which behaves in a similar manner in its responses to electron transfer inhibitors, as the respiratory chain found in intact mitochondria (9-11).

Redox potential measurements on both 'in situ' and isolated components of the respiratory chain have enabled the presentation of the electron carriers on a potential diagram (Fig. 1.3) (12). In Fig. 1.3 it is shown, that when the components associated with three levels of mid-potentials are classed or grouped together, there are three spans or gaps in the chain in which relatively large decreases in free energy occurs, each sufficient to provide the energy for the formation of ATP from ADP and inorganic phosphate. The sites are designated Site I, II and III and represents the span between NADH and coenzyme Q; cyt.b and cyt.c and cyt.a and oxygen respectively. Chance, in his review of 1972 (12), suggested that the three groups of electron carriers having fixed mid-potentials (or isopotential pool) were linked with one another by means of energy-transducing carriers of variable potential. The energy-transducing carriers of site I, II and III are proposed to be $(Fe\text{-}S)_{N_2}$, cyt.b_T and cyt.a_3 respectively, based on the findings that their mid-potentials were ATP and pH dependent (4, 12, 13). The isopotential pools functions as isopotential redox ballasts involving rapid equilibriating electron transfer reactions that are independent of energy coupling reactions. The respiratory chain therefore acts by an energy transduction process that



I = NADH-ubiquinone reductase complex which catalyses the reaction: $NADH + Q + H^+ \rightarrow NAD^+ + QH_2$

II = Succinic ubiquinone reductase complex which catalyses the reaction: $Succinate + Q \rightarrow Fumarate + QH_2$

III = Ubiquinol cytochrome c reductase complex which catalyses the reaction: QH_2 + 2 ferricyt. c → Q + 2 ferrocyt. c

IV = Cytochrome c oxidase complex which catalyses the reaction: 2 ferrocyt. c + $2H^+ + \frac{1}{2}O_2 \rightarrow$ 2 ferricyt. c + H_2O

Fig. 1.2 The four complexes and their sequential arrangement in the respiratory chain (9).

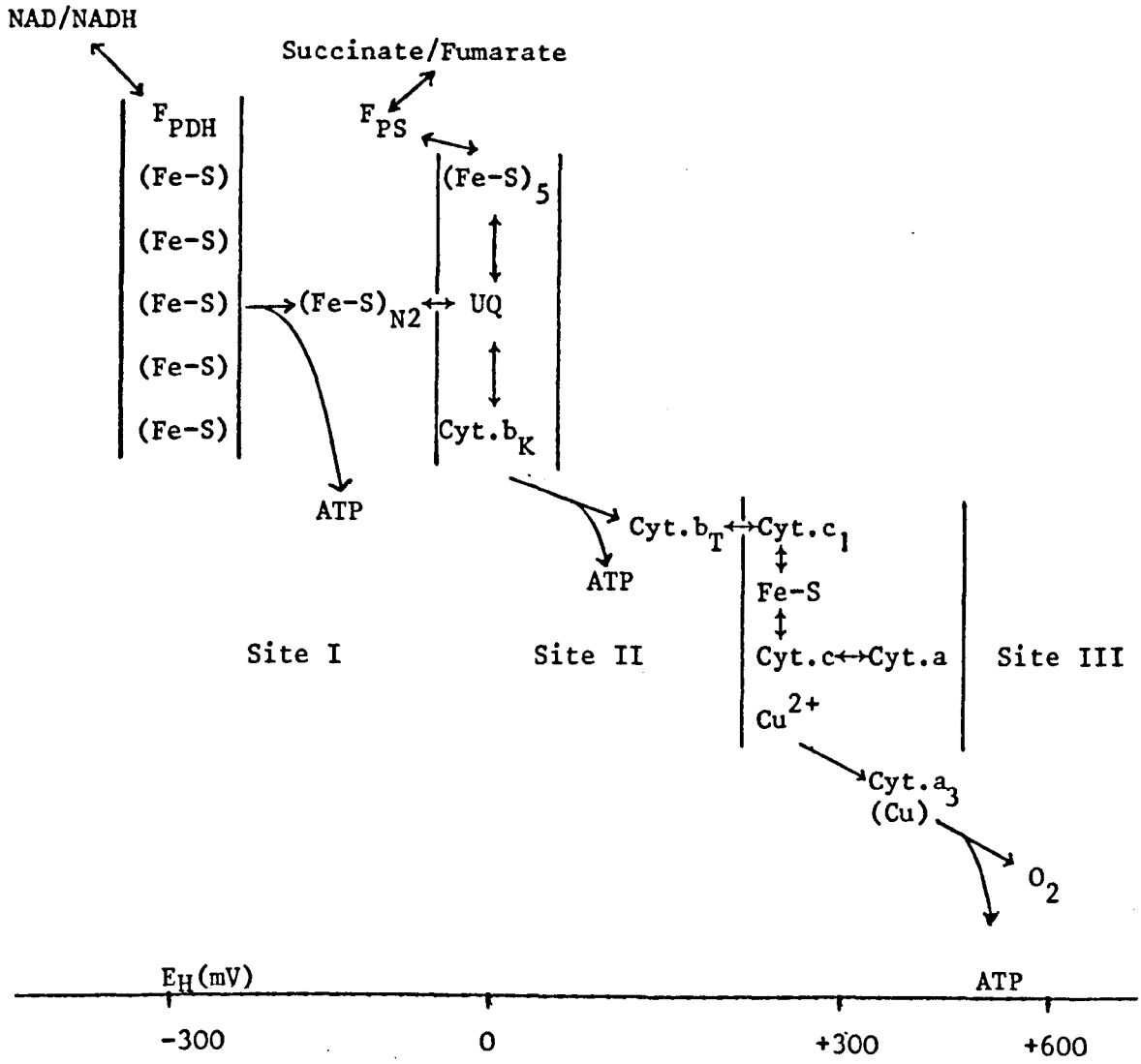


Fig. 1.3 The electron carriers of the respiratory chain arranged as a group of fixed potential (or isopotential 'pools') and individual components of variable mid-potentials (b_T, (a_3) and Z). The other components are the same as in Fig. 1.1, from [Modified from Chance 1972 (12)]. (Z = $(Fe-S)_{N2}$)

operates through a series of quasi-equilibrium steps. In the case of cyt.b_T for example, there are four species; oxidised high mid-potential, oxidised low mid-potential, reduced high mid-potential and reduced low mid-potential. b_T in its low mid-potential form can only react with group II carriers and in its high mid-potential form can only react with group III carriers.

The actual site of ATP synthesis is now known to reside on the multi-enzyme ATP synthase complex, which although located in the inner membrane of the mitochondria, is not directly linked to the respitatory chain. It is therefore important, to consider the problem of how energy from the respiratory chain is made available to the ATP synthase in such a form that it drives ATP synthesis.

1.3 MECHANISMS OF ENERGY TRANSDUCTION

Currently, the theories available to describe the phenomenon of oxidative phosphorylation, falls mainly into three broad categories:

(a) chemical,

(b) conformational, and

(c) protonic.

All the theories propose that the respiratory chain generates a high energy intermediate which can drive ATP synthesis, transhydrogenation, ion transport as well as reversed electron transport. However, they differ in the nature of high energy intermediate proposed.

1.4 THE CHEMICAL HYPOTHESIS

The chemical hypothesis as originally proposed [Slater 1953 (14)] was based on a mechanism of substrate level phosphorylation (15). The central feature of the hypothesis is that the respiratory chain generates high energy chemical intermediates which can be used to drive ATP synthesis or other energy-linked reactions. Using the notation of Chance and Williams (16), the hypothesis can be represented by the following sequence of reactions:

1. $AH_2 + B + I \rightleftharpoons A \sim I + BH_2$
2. $A \sim I + X \rightleftharpoons A + X \sim I$
3. $X \sim I + Pi \rightleftharpoons X \sim P + I$
4. $X \sim P + ADP \rightleftharpoons X + ATP$

AH_2 and B are two adjacent respiratory carriers and '$\sim$' refers to a high energy bond (probably anhydride or thioester bond). The nature of X and I is unknown. $X \sim I$ is regarded as the common energy transducing intermediate. This is considered as being the target of action of uncoupling agents, which, directly or indirectly, catalyse its hydrolysis (19, 20). Oligomycin is thought to block reaction 3, and transhydrogenation and ion transport are both driven by utilisation of $X \sim I$, produced either by respiration or by ATP (17-20). Evidence for the chemical hypothesis is largely based on the actions of inhibitors like oligomycin, and on the fact that partial reactions such as transhydrogenation and reversed electron transfer can be driven either by energy deriving from respiration or ATP. However, there are a number of disquieting features in the chemical hypothesis. It provides no explanation for:

(a) the apparent dependence of oxidative phosphorylation on the integrity of the mitochondrial membrane

structure; and

(b) the fact that a wide range of molecules of widely different structures will uncouple respiration from phosphorylation.

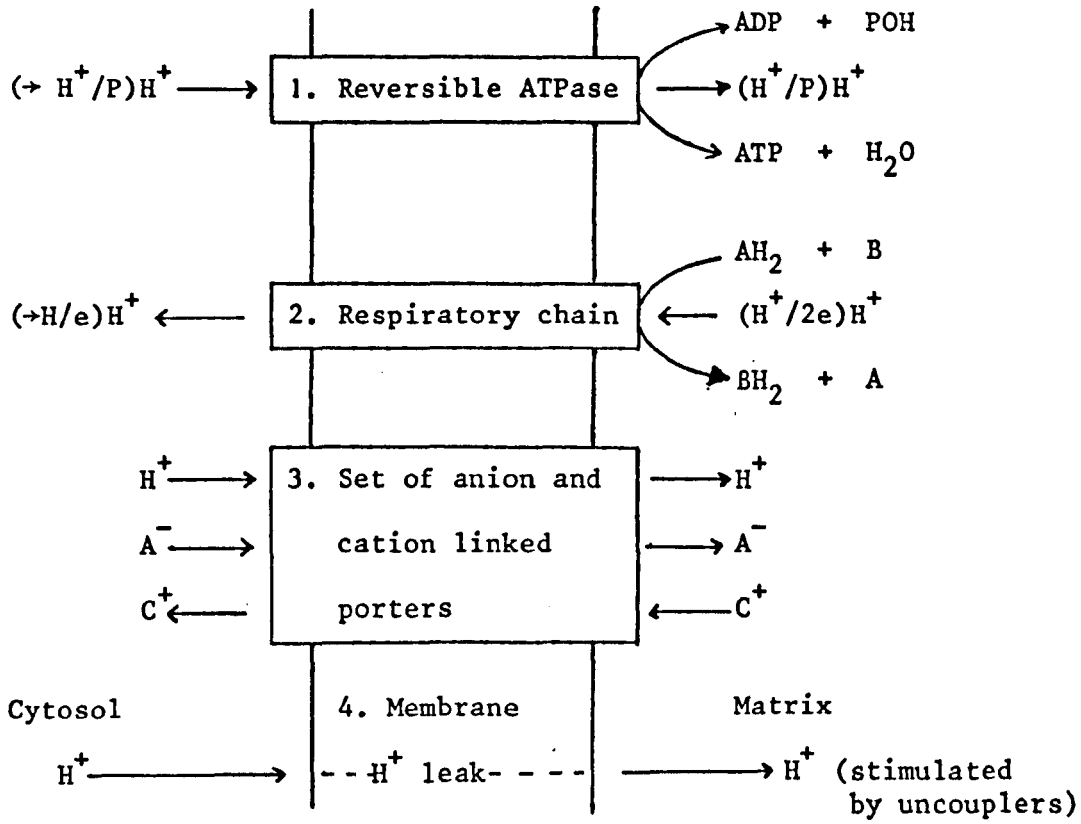
The inability to isolate and identify the proposed high energy intermediate adds considerable complication to the chemical hypothesis (21, 22).

1.5 THE CHEMIOSMOTIC HYPOTHESIS

The chemiosmotic hypothesis, proposed by Mitchell in 1961 (23) is based on the following four main postulates which are summarised in Fig. 1.4:

1. The ATP synthase is a chemiosmotic membrane-located reversible protonmotive ATPase, having characteristic H^+/P stoichiometry.
2. The respiratory chain is a membrane located vectorial metabolic proton translocating system, having a characteristic $H^+/2e$ stoichiometry and having the same polarity of proton translocation across the membrane for normal forward redox activity as the ATPase for ATP hydrolysis.
3. There are proton (or hydroxyl) linked solute porter systems for osmotic stabilisation and metabolite transport.
4. Systems 1-3 are 'plugged' through a topologically closed insulating membrane, called the coupling membrane, which is impermeable to H^+ and OH^-.

Fig. 1.4 Fundamental postulates of chemiosmosis (23-28)

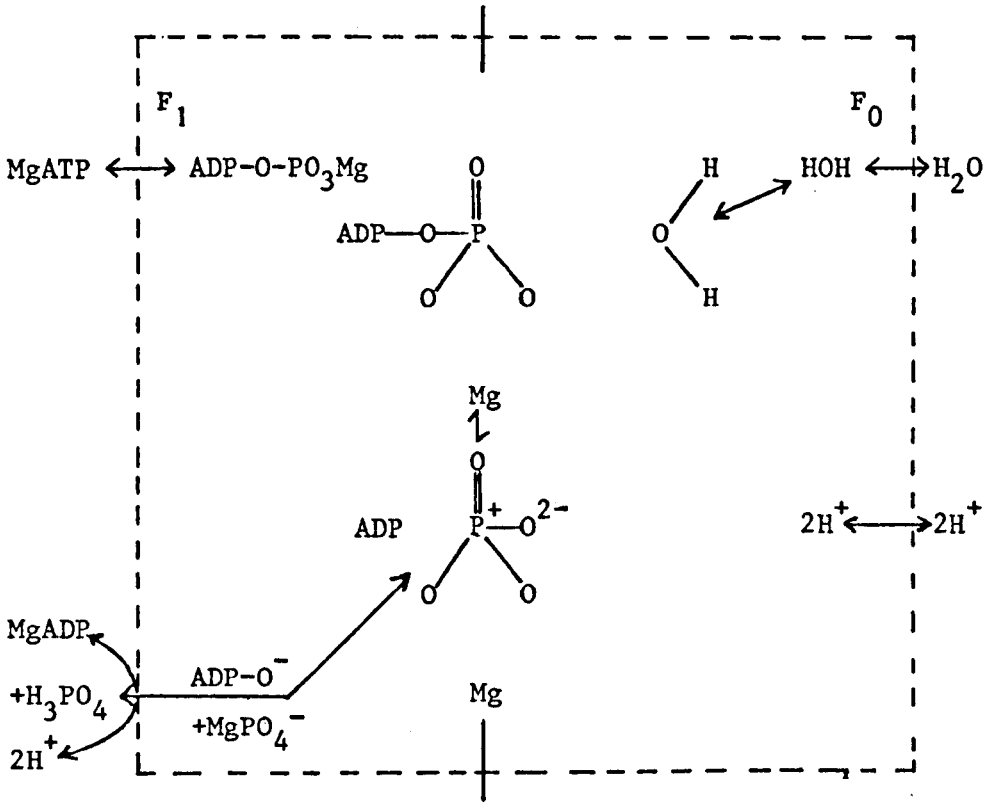


Mitchell pointed out that, since the basis of oxidative phosphorylation was a dehydration reaction to form an acid anhydride link, a system which causes the removal of water from the active site of the ATPase would favour the formation of ATP (Fig. 1.5). The system Mitchell suggested for causing this dehydration consisted of an anisotropically organised respiratory chain consisting of alternating electron carriers and hydrogen carriers (Fig. 1.6) and an anisotropic membrane ATPase. He proposed that phosphorylation was linked to electron transport by a protonic electrical potential set up across the coupling membrane by the action of the respiratory chain which pump protons from one side of the membrane to the other. The protonic electrical potential cause the removal of (H_2O as) H^+ in one direction and OH^- in the opposite direction from the active

centre of the ATPase (Fig. 1.5). The protonic electrical potential or protonmotice force (Δp) is the sum of the pH gradient (ΔpH) and the membrane potential ($\Delta\psi$) according to the equation (23-28):

$$\Delta p = \Delta\psi - z\Delta pH.$$

Fig. 1.5 Proton translocation by the F_1F_0 ATPase (26)

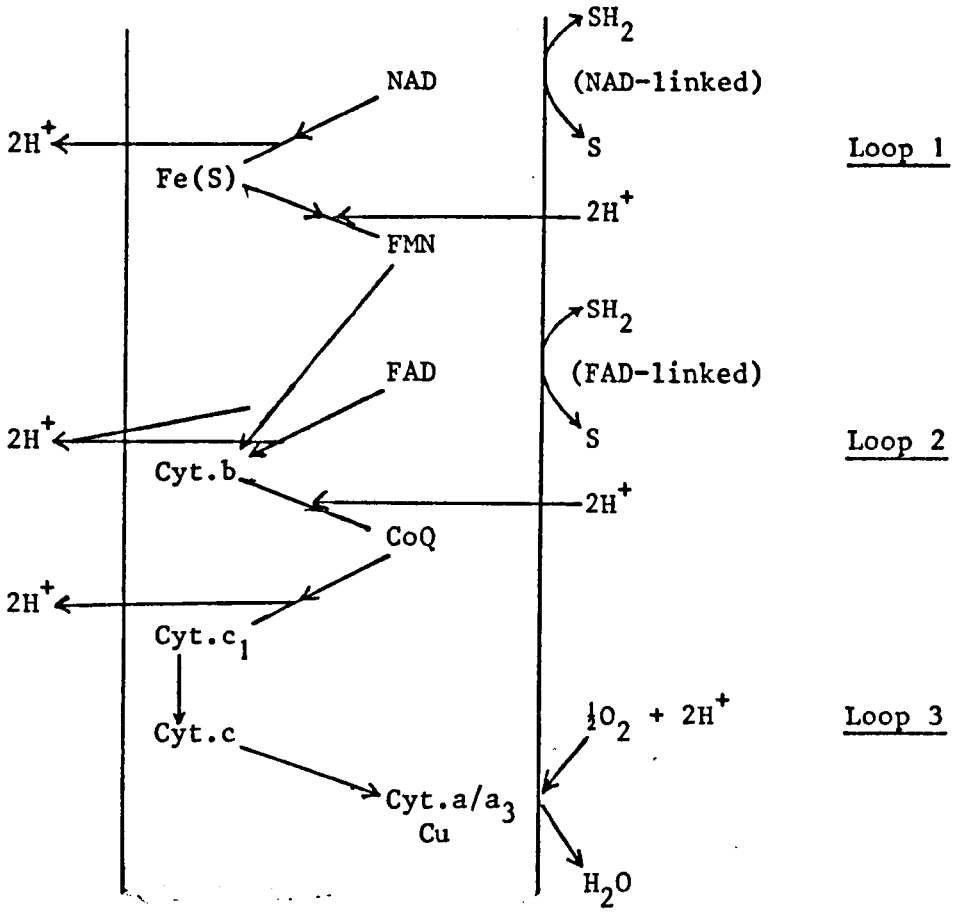


When the membrane is not leaky and most of the proton current generated by the redox system passes back through the reversible ATPase, there will be stoichiometric coupling between oxidation-reduction and phosphorylation. The P/O ($P/2e^-$) quotient of the overall process is given by the $\rightarrow H^+/2e^-$ quotient of the redox system divided by the $\rightarrow H^+/P$ quotient of the ATPase, that is:

$$P/2e^- = (\rightarrow H^+/2e)/(\rightarrow H^+/P).$$

A short circuiting proton pathway across the membrane [as provided by uncouplers (23-28)] would, collapse Δp, uncouple phosphorylation, and allow redox activity to accelerate.

Fig. 1.6 Proton translocating redox loops (26)



Detailed arrangement of the respiratory carriers according to Mitchell. The scheme provides for the translocation of $6H^+/O$ for the oxidation NAD-linked substrates and $4H^+/O$ for succinate oxidation (29).

With the chemiosmotic hypothesis, Mitchell, circumvented two of the major objections to the chemical hypothesis. Firstly, the integrity of the coupling membrane is essential to maintain a membrane potential and pH gradient. Secondly, there is no need to postulate the existence of energy-rich intermediates to couple respiration and phosphorylation since the ATPase transfers Pi directly to ADP.

That the chemiosmotic hypothesis has gained such wide acceptance in studies on membrane bioenergetics, may be ascribed to evidence derived from studies on mitochondria, submitochondrial

particles, and reconstituted vesicles, summarised below.

1. Vectorial, transmembrane movement of H^+ and other ions accompany both respiration and ATP hydrolysis (30-35).
2. Most if not all the various types of energy transducing systems can generate a proton gradient and/or a membrane potential across the membrane in which they are located (35).
3. Energy transfer between energy-transducing systems located in the same membrane can take place via a proton gradient and/or membrane potential (35).
4. The action of some uncouplers depends on their ability to dissipate the transmembrane proton gradient, since almost all are lipophilic weak acids (35-38).
5. Artificially generated electrochemical proton gradients are kinetically competent in driving ATP synthesis (39, 40).

Although the chemiosmotic hypothesis offer a generally acceptable framework for energy transduction, the proposed mechanisms for H^+ translocation (Fig. 1.6) and ATP synthesis (Fig. 1.5) are no less hypothetical than those involving high energy intermediate (14). Mitchell's suggestion that the proton gradient is generated by the action of H^+ translocating 'redox loops', formed by alternating hydrogen carriers and electron carriers of the respiratory chain (Fig. 1.6), has been refuted by a number of workers (30). Harmon et al. have pointed out, that although complexes I and III contain both hydrogen (NAD, FMN, CoQ) and electron carriers (Fe-S, cyt.b and c), (unlike complex IV, which contains only electron carriers), they do not form transmembrane loops (30, 40). The lack of support for his 'redox loop' concept, led Mitchell

to propose his 'protonmotive ubiquinone cycle' (Q cycle) for H^+ translocation [see (42, 43) for reviews]. However, the 'Q cycle' requires the movement of Q and QH_2 across the membrane, and a certain distribution of b cytochromes across the membrane. Available evidence suggests that these situations are unlikely (30). In the case of the ATPase system, much evidence has been compiled, which indicates that the F_0F_1 ATPase complex may function as a H^+ translocator (44, 45). However, the direct interaction of Pi and ADP, with the intermediate formation of a phosphonium ion $[PO_3]^+$, to yield ATP at the catalytic site (Fig. 1.5) proposed by Mitchell (46, 47) has been questioned on theoretical grounds by Boyer and Williams (48, 49).

The mechanisms by which the respiratory chain and the ATPase complex transport H^+ and the H^+/site quotient are matters of considerable controversy. In both cases the problem concerns whether proton translocation proceeds directly, involving the catalytic site(s) of the enzyme, or whether it proceeds indirectly <u>via</u> a 'proton pump'. Information deriving from investigation of the membrane topology of the catalysts involved and measurement of the $\rightarrow H^+/2e$ and $\rightarrow H^+$/ATP ratios, support an indirect mechanism involving proton pumps (50). Direct coupling redox loops would require a stoichiometry of two H^+ translocated per two electron translocated through each coupling site (i.e., $\rightarrow H^+$/site = 2). Similarly, direct coupling between the ATPase and the transmembrane 'proton well', of the type postulated by Mitchell (26), predicts a stoichiometry of two H^+ per molecule of ATP synthesised (i.e., $\rightarrow H^+$/site = 2). Results supporting these predictions have been reported by Mitchell and Moyle, who have shown that electron transport from NADH to oxygen cause the translocation of $6H^+$ (23-28). However, other

workers have obtained significantly higher $\rightarrow H^+$/site ratios, often exceeding 3, for both the electron transport and the ATPase systems (35, 51-54). These stoichiometries ($\rightarrow H^+$/site ratio $\geq$ 3) are not compatible with the proposed 'redox loop' and 'proton well' mechanisms, but can be readily accommodated by a mechanism involving proton pumps (32). The reported transmembrane location of Fe.S centres 1 and 2 in complex I (55-56) or the two b cytochromes in the case of complex III (55-58) is equally compatible with the occurrence of transmembrane proton pumps driven by nonvectorial electron transfer catalyst. However, the mechanism by which protons are translocated through the 'pumps' is not defined.

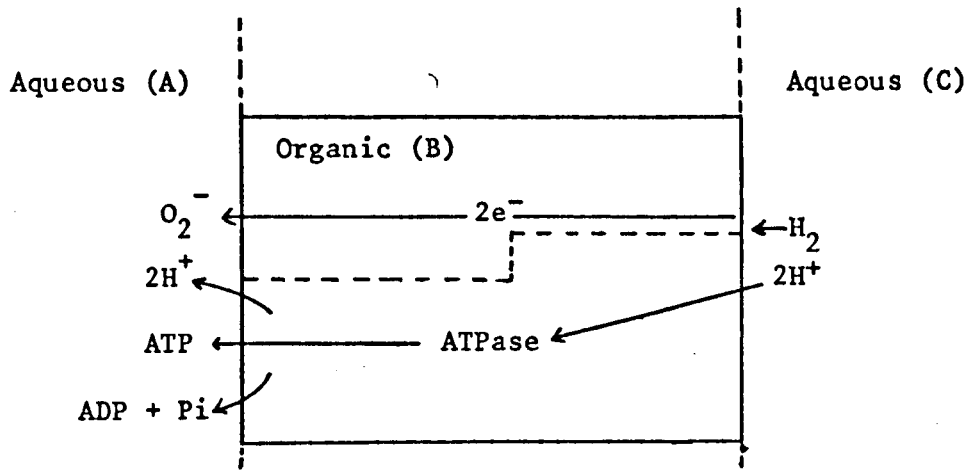
1.6 LOCALISED PROTON HYPOTHESES

Two alternative hypothesis envisaging proton coupling have been put forward to explain the inadequacies of the chemiosmotic hypothesis. They are the 'Localised proton hypothesis' of Williams (59-60) and the 'Electrodic hypothesis' of Kell (61). Only a summary of these hypotheses will be presented, because they have been presented, only as logical models for discussion [for in depth reviews see ref. (219-221)].

In the 'localised proton hypothesis', Williams proposes that the protons produced by the respiratory chain, and which are responsible for ATP synthesis, are not pumped across the membrane as proposed by Mitchell (23-28), but remain in the membrane where they lower the activity of the "solvent water" in the ATPase region (Fig. 1.7). The energy of the redox reactions is thus transferred to the ATPase complex as anhydrous (or partially hydrated) H^+, which moves in the membrane through a channel of fixed water molecules,

which is connected to the ATPase (Fig. 1.8).

Fig. 1.7 The localised proton model for ATP synthesis (59)



The solid lines (box) represent a very hydrophobic particle within which ADP and Pi are condensed to ATP by the ATPase.

Thus during the synthesis of ATP from ADP and Pi the H^+ moves so as to pull H_2O molecules from the polyphosphate condensation site into the diffusion channel [Fig. 1.8 (60-63)].

The obvious advantages of Williams' hypothesis over the chemiosmotic hypothesis are that it offers:

(a) more efficient energy utilisation,

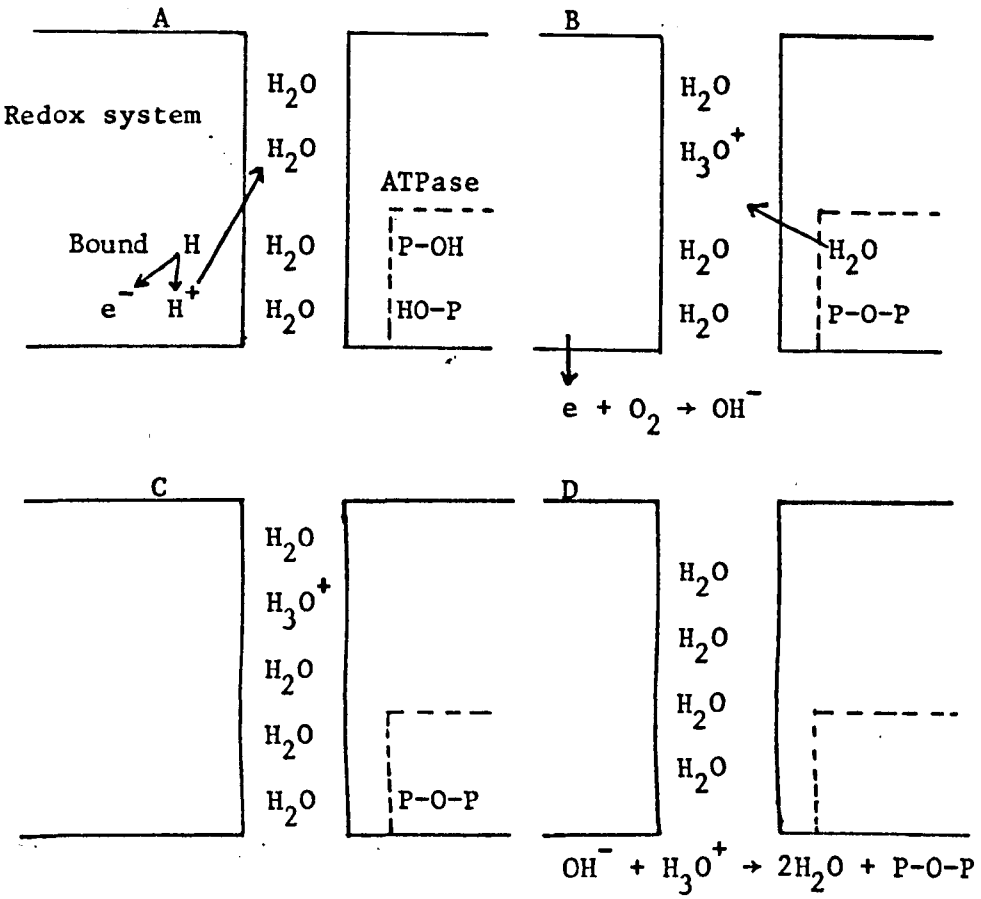
(b) better control, and

(c) discriminatory coupling of energy to different processes.

However, although a certain degree of localisation of H^+ takes place between the electron transport and ATPase system (64-65), Mitchell has pointed out that Williams' hypothesis is very limiting and not experimentally falsifiable (28). In particular, it lacks explanation for transport, and because it lacks stoichiometry, it cannot be used to explain the experimentally observed P/O quotients.

In his 'electrodic hypothesis', Kell proposes that the protons that are responsible for ATP synthesis are pumped across

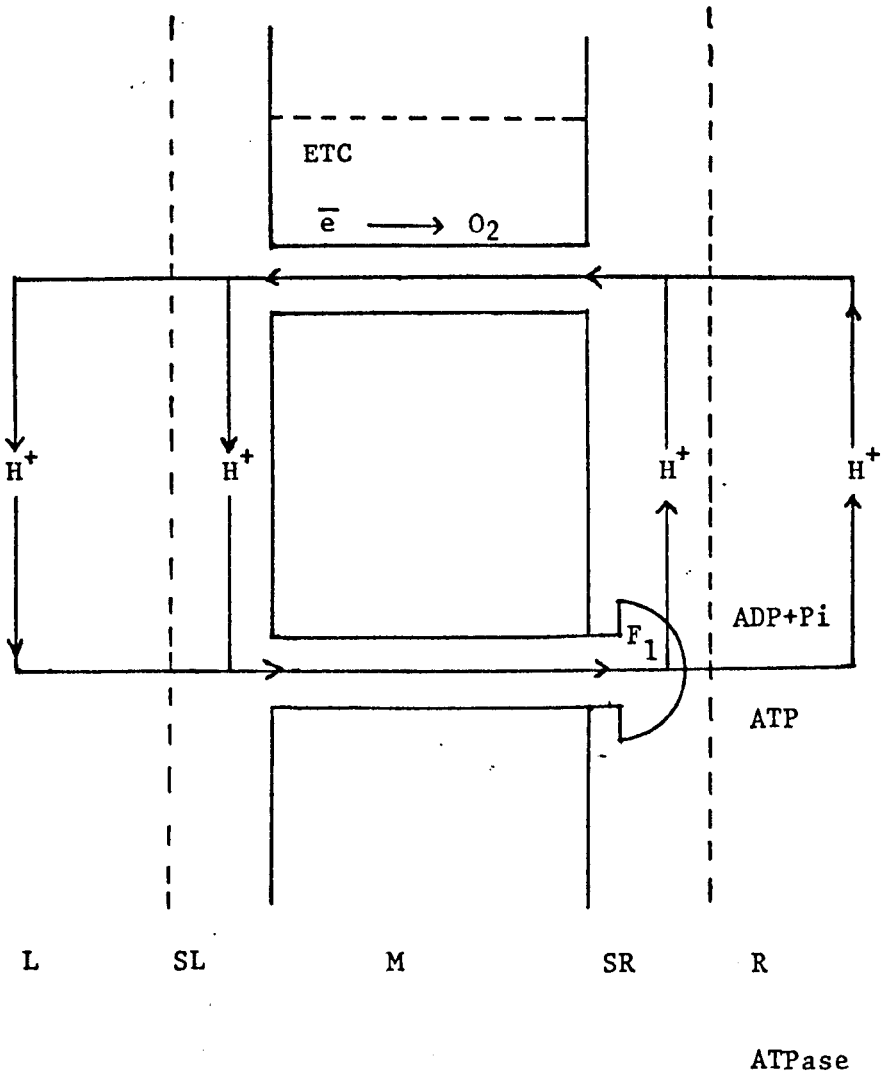
Fig. 1.8 Indirect localised proton coupling model (63)



An open channel model is shown so that it could in principle be linked to chemiosmotic gradient, although this is not required in the hypothesis.

the membrane from one S-phase (SR-phase) (membrane-liquid interphase) to the other (SL-phase) by the electron transfer complex (Fig. 1.9). The protons then flow along the SL-phase to the ATPase through which they re-enter the original SR-phase completing the protonic circuit. The flow of protons along the S-phase is 'conducted' by 'structured water molecules' (or membrane water) which allows H^+ tunnelling (66, 67). The movement of protons between the S-phases results in charging the membrane surfaces relative to the bulk aqueous phases, and it is the potential drop across the membrane/solution (electrode/electrolyte) intersurface which drives ATP synthesis.

Fig. 1.9 Electrodic hypothesis (61)



A typical protic circuit. The diagram represents a phospholipid membrane phase (M) separating two aqueous phases L and R repsectively. Interphase SL and SR exist between the M phase and the two aqueous phases. The M phase contains the protonmotive electrontransport complex (FTC) and protonmotive ATPase of the appropriate polarities, and the radial and lateral flow of the proton current, i.e., a two-dimensional proton current flow, between them is indicated by the arrows. Adopted from Kell (61).

Whilst the postulates of Kell (61) are in broad agreement with the proposals of Williams (59-63), the electrodic hypothesis is chemiosmotic because a transmembrane-phase H^+ gradient is involved. Kell has layed particular emphasis on localised interphase phenomena based on generally accepted physical, chemical and electrochemical principles, and has provided explanations for ion transport, and the action of uncouplers (61, 68, 69). However, like the chemiosmotic and 'localised proton' hypotheses, it lacks detailed molecular mechanisms for H^+ translocation and ATP synthesis.

1.7 CONFORMATIONAL HYPOTHESIS

The conformational hypothesis in its original form [Boyer 1965 (72)] proposed that the energy generated by electron transport, was conserved in a 'high-energy' conformational state of the respiratory carrier. The energy inherent in this conformation being used to drive ATP synthesis:

$$AH_2 + B \rightleftarrows A^* + BH_2$$

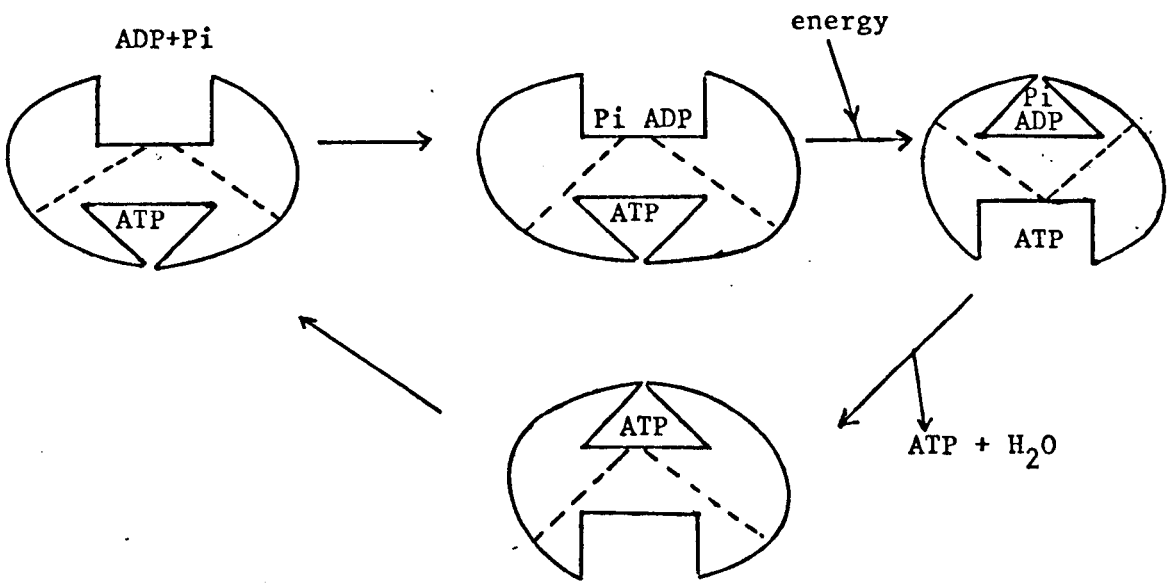
$$A^* + ADP + Pi \rightleftarrows A + ATP + H_2O$$

Support for this hypothesis is obtained from Green (73) and Hackenbrock (74) who observed that the structure of the mitochondria was energy dependent, and from the observations that electron flow in bacteria, chloroplast and mitochondria is accompanied by changes in the conformation of their F_1 ATPase (70, 75-77). However, little compelling evidence has been found to support direct interaction between the ATPase and the redox centres. Mitchell (28) has pointed out that such an interaction does not occur.

In the new conformational hypothesis Boyer and Slater

proposed that ATP synthesis occursat the active site of the ATPase complex prior to the input of energy; which is necessary, only, to bring about the release of ATP from the active site, via an energy dependent conformational change [Fig. 1.10, (70, 79)]. This hypothesis was based on the finding that the exchangeability of adenine nucleotides bound to F_1 was dependent on the flow of electrons (70, 79) and on the insensitivity of the anhydride formation to uncouplers of oxidative phosphorylation (80). These findings although intimately correlating with the suggested molecular explanation for exchange reactions; namely that they result from the dynamic reversal of ATP formation, are difficult to reconcile with Mitchell's proposal that the protons are directly involved in the anhydride formation (28).

Fig. 1.10 New conformational coupling model (70)



Dotted line represents changing conformational state due to energisation. The energy is derived from electron transport.

1.8 THE MITOCHONDRIAL ATPase COMPLEX

The mitochondrial ATPase complex appears to be one of the most complex enzyme systems known to date, performing 'in situ' a number of reactions such as ATP-Pi exchange, ATP synthesis and hydrolysis. These reactions are affected by a variety of inhibitors including oligomycin, DCCD, venturicidin and trialkyltins. The inhibitor studies have proved to be very useful in trying to determine the subunit/activity relationships in the complex, and in some cases, have proved to be instrumental in the proposal of new mechanisms for coupling ATP synthesis and respiration. Inhibitor studies have also indicated that the ATPase complex is at least bifunctional. Of interest are those experiments which show that some inhibitors such as AMP-P-NP are capable of either activating or inhibiting ATP hydrolysis without altering ATP synthesis (81). Work presented in Chapter 2 of this thesis has shown that such a differential effect on ATP synthesis and ATP hydrolysis can be demonstrated with trialkyltin compounds in beef heart mitochondria. A brief description of the ATPase will now be presented. For more in depth reviews see references (81-88).

The mitochondrial ATPase complex is located exclusively in the inner membrane where it protrudes into the matrix space (82). In general, it accounts for about 2% of the mitochondrial mass (81). The 'complete' ATPase complex (Fig. 1.11A) is depicted by many authors as consisting of four major components: a headpiece called F_1 which is water soluble, catalyses the hydrolysis of nucleoside triphosphates and binds the inhibitor aurovertin; a basepiece or membrane sector, which is detergent soluble and binds the inhibitors oligomycin and DCCD; a stalk connecting the headpiece and basepiece; and one or more small peptide inhibitors of ATP hydrolytic activity.

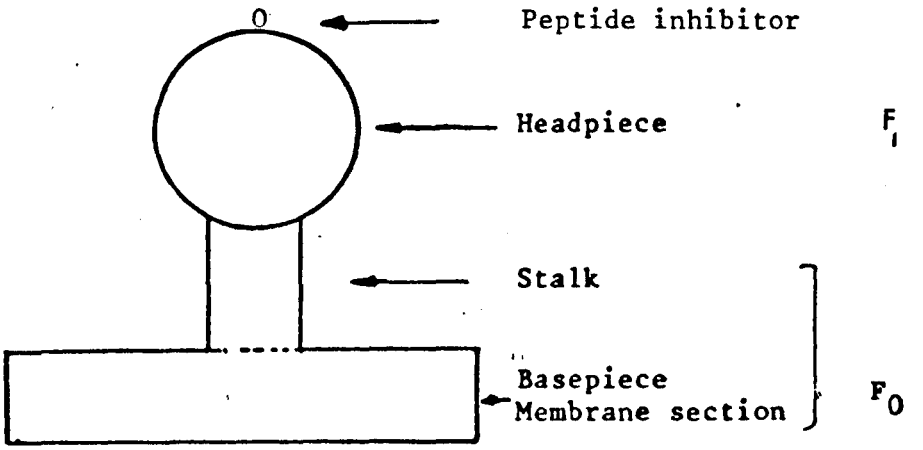


Fig. 1.11A Schematic representation of the mitochondrial ATPase complex (81)

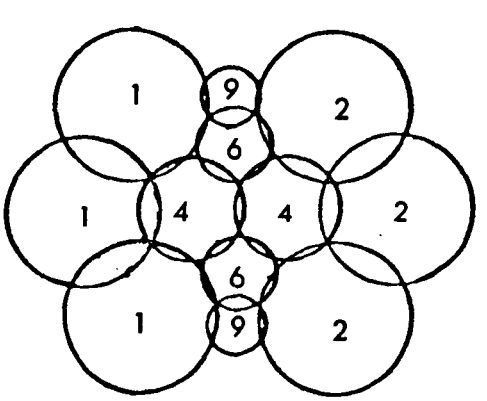


Fig. 1.11B Plan view

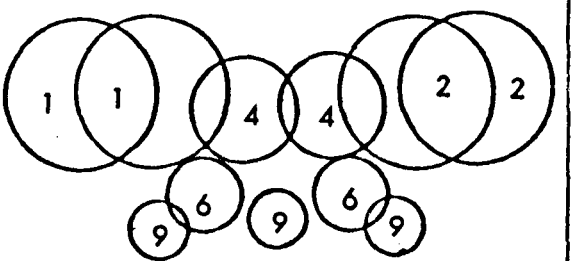


Fig. 1.11C Side view

Three dimensional structure of F_1F_0-ATPase (109)

The numbers on Fig. 1.11B and C refer to the subunit type. The position of the other subunits of the ATPase complex, notably 3, 5, 7 and 8 is as yet unidentified.

The stalk and basepiece are referred to collectively as F_0.

The F_1F_0 ATPase complex (or complex V) of beef heart mitochondria has been prepared by a variety of methods (81, 87-89), all of which involves solubilisation of the inner mitochondrial membrane with chaotrophic detergents such as Triton-X-100 and deoxycholic acid. These treatments result in severe delipidation of the final purified form of the F_1F_0 ATPase complex, and the expression of its maximal enzymic activities is dependent on the presence of exogenous lipids (81). The purified complex has an estimated molecular weight of 460-480,000 and contains 8-12 different subunits (84, 88-90). Hatefi et al. have recently shown that the F_1F_0 ATPase complex of beef heart mitochondria contained at least 12 subunits, most of which have been tentatively described on a functional basis in Table 1.1 (84).

The purified F_1F_0 ATPase complex catalyses ATP hydrolysis and ATP-Pi exchange; however maximal catalytic activity is dependent on the addition of exogenous phospholipids (81). Both the ATP-Pi exchange and ATP hydrolytic activities are sensitive to inhibitors such as oligomycin, venturicidin, trialkyltins and DCCD. The F_1F_0 ATPase complex appears to be divided into two functionally dissimilar portions: the hydrophylic F_1ATPase which protrudes from the inner membrane into the matrix; and the F_0 portion which is hydrophobic in nature and is buried in the inner membrane, both of which, have been isolated in purified form (92, 93, 104, 105).

The F_1 ATPase has been isolated from the inner mitochondrial membrane by a variety of methods, including sonication (92, 93) and chloroform treatment (94). The enzyme prepared from beef heart mitochondria has a molecular weight of 347-360,000 and has been shown using SDS-gel electrophoresis to be composed of five different

Table 1.1

Subunit M.W.	Identity of subunits of the F_1F_0 ATPase Complex (84)
53,000	α subunit F_1 ATPase
50,000	β subunit F_1 ATPase
33,000	γ subunit F_1 ATPase
30,000	uncoupler binding protein
23,000	F_0 component; pantotheine binding protein (86)
22,000	oligomycin sensitivity conferring protein (OSCP)
15,000	δ subunit F_1 ATPase
12,000	DCCD binding protein
8,000	coupling factor F_6 (85)
11-13,000	ATPase inhibitor or coupling factor B (87, 91)
6,000	ε subunit F_1 ATPase

Numbers in brackets are references; M.W. = molecular weight.

subunits (84, 93). These subunits are designated α, β, γ, δ and ε in Table 1.1. Measurement of the subunit stoichiometry, using dye staining and amino acid frequency methods, indicate that there are at least 9-10 polypeptide components per F_1 ATPase molecule (92, 93). Senior has suggested a subunit stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ for the beef heart enzyme (92) and a similar stoichiometry has been proposed for the rat liver enzyme by Catterall and Pedersen (95) and for the yeast enzyme, by Tzagoloff and Meagher (90). An additional 10,000 molecular weight subunit found in certain preparations of F_1 ATPase has been proposed to be the 'ATPase inhibitor protein' which regulates the ATPase activity 'in vivo' (96).

Purified F_1 ATPase exhibits only ATP hydrolytic activity

and is insensitive to inhibitors such as oligomycin, DCCD, venturicidin and trialkyltin compounds. However, F_1 ATPase is inhibited by aurovertin and efrapeptin. The true substrate of the enzyme is the Mg ATP complex; however, inosine and uridine triphosphate can also act as substrates (97). 'In situ' the F_1 ATPase catalyse ATP synthesis, hydrolysis and ATP-dependent reactions such as transhydrogenation. This has been demonstrated by a series of reconstitution experiments, in which F_1 ATPase was shown to couple ATP synthesis and ATP-dependent activities in F_1-ATPase depleted membrane vesicles (81).

1.9 NUCLEOTIDE BINDING SITES

The binding of nucleotides to F_1 has been studied extensively using many different techniques (98-101). Slater et al. have suggested that there are at least seven ATP and ADP binding sites on each F_1 ATPase molecule and that these could characterise into four types, designated type I, II, III and IV (99). Types I and II bind ATP and ADP respectively, very strongly and are thought to be involved in ATP synthesis, since their binding affinities are weakened in membrane bound F_1, when the membrane is energised by electron flow through the respiratory chain (99, 102, 103). There are two type III binding sites, one on each β-subunit, and these are proposed to be the catalytic site for ATP hydrolysis. One type IV binding site is found on each α-subunit and these sites are proposed to be allosteric anion binding sites (99, 101). Based on these binding studies, Slater et al. (99) have suggested that there are at least two catalytic sites on the F_1 ATPase, one specialised for ATP synthesis and the other specialised for ATP-

dependent reactions. Support of this suggestion comes from the differential effects of aurovertin (124), AMP-PNP (125), quercetin (126) and the inhibitor protein of Pullman and Munroy (127) on oxidative phosphorylation and ATP dependent reactions.

It is well known that a ten-fold greater concentration of aurovertin is required to inhibit ATPase than to inhibit oxidative phosphorylation. Chang and Penefsky (128) have suggested that there are two aurovertin binding sites, one involved in the inhibition of oxidative phosphorylation, and the other in the inhibition of the ATPase.

The F_0 component of the F_1F_0 ATPase complex unlike the F_1 ATPase component is not catalytically active. However, it is required for the expression of oligomycin sensitivity and ATP-dependent reactions. The F_0 sector is supposed to be the region in which the high energy state '$\sim$', generated by coupled electron transport, is transduced to activate inorganic phosphate. In the chemiosmotic hypothesis the F_0 component is considered to be a proton conducting well spanning the inner membrane, through which protons flow to the active site of the F_1 ATPase. This view is supported by the findings that the increased proton permeability in submitochondrial particles depleted of F_1 can be inhibited by oligomycin, which binds to the F_0 component (104, 105).

The F_0 component contains 5-7 different subunits, the most well characterised of which are, the DCCD binding protein, the oligomycin sensitivity conferring protein (OSCP) and coupling factor F_6 (see Table 1.1). The OSCP binds directly to F_1 (in a 1:1 ratio) and to the membrane (106, 107). F_6 appears to facilitate the binding of F_1 to the membrane by binding OSCP (84, 108). The OSCP form the stalk of the F_1F_0 ATPase complex

shown in Fig. 1.11.

Although the distribution of subunits between the F_1 and F_0 portions of the F_1F_0 ATPase complex has been known since 1973 (92), little is known about the spatial arrangement of these subunits in the F_1 or F_1F_0 ATPase complexes. However, Enns and Criddle have recently investigated the 3-dimensional arrangement of these subunits in the F_1 and F_1F_0 ATPase complexes, using cleavable and non-cleavable cross-linking reagents (e.g. mercaptobutyriimidate) and 2-dimensional SDS-gel electrophoresis (109). From these investigations Enns and Criddle found that the following pattern of cross-linking occurs; β-β, α-α, α-β, γ-ε. The β and α subunits were also found to link with each of the other subunits. From the results of these studies Enns and Criddle proposed the structure presented in Fig. 1.11B, and 1.11C as a diagramatic representation of the 3-dimensional structure of the F_1F_0 ATPase complex (109).

1.10 A GENETIC APPROACH TO OXIDATIVE PHOSPHORYLATION

As outlined in the preceding sections of this Chapter, the chemical transformations occurring in oxidative phosphorylation have been difficult to resolve with the usual biochemical techniques. The fundamental problem is that the catalytic units are often an integral part of the membrane structure. This observation has prompted a number of laboratories to tackle the problem of energy conservation by means of a mutant approach. Much of the work to date has been carried out on the eucaryote, Saccharomyces cerevisiae

Kovac has reviewed the advantages of yeast as suitable organisms for biochemical genetic studies of oxidative phosphorylation (110). Briefly, its main advantages are:

1. it possesses mitochondria with properties very similar to mammalian mitochondria;
2. its biochemistry, genetics and cytology are known in great detail;
3. and it can survive major genetic lesions affecting the mitochondria, since it can grow on fermentable substrates.

He concluded that the most suitable yeast for these studies is the strain Saccharomyces cerevisiae, largely because its biochemistry, genetics and cytology are the most well documented.

The basic approach has been to make use of the fact that mitochondria are semi-autonomous organelles possessing their own DNA (mtDNA) and protein synthesising systems. Moreover, mitochondrial protein synthesis can be specifically inhibited by chloramphenicol and erythromycin but not by cycloheximide which inhibits cytoplasmic protein synthesis (111, 112). This has enabled Schatz et al. (113) and Tzagoloff et al. (114) to produce results which indicate that three subunits of cytochrome oxidase, two subunits of ubiquinone - cytochrome c reductase and four subunits of the ATPase system are mitochondrially synthesised.

The first type of mutant to be actively investigated were the respiratory deficient mutants known as petites. Petites can form spontaneously, but they are usually induced by the action intercalating drugs such as ethidium bromide. They are usually characterised by the fact that they form smaller than normal size colonies on media containing glucose as energy source, their inability to grow on non-fermentable substrates, and their cyanide insensitivity (115). The petite mutation represents a major deletion of the mtDNA and is expressed as an inability to carry out

mitochondrial protein synthesis (116). Schatz (113) and Tzagoloff and Meagher (90) have shown that the ATPase present in petite mitochondria is F_1-ATPase and that the F_0 components were absent. Mitochondria of petite mutants do not catalyse an ATP-Pi exchange reaction and are not energised by ATP as is shown by the lack of fluorescence with ANS (110). The conclusion from these experiments is that the F_0 subunits are necessary for the ATPase to participate in energy conservation and transduction.

An alternative approach to the isolation of mutants with altered components of the energy conservation system has been adopted by Griffiths (117-121). Mutants are selected for growth on non-fermentable substrate in the presence of inhibitors of oxidative phosphorylation. Any such mutants not due to cell or mitochondrial membrane impermeability or detoxification may be due to a modified ATPase complex. Mutants resistant to oligomycin (118), triethyltin (119, 121) and venturicidin (117, 120) have been isolated. The oligomycin-resistant mutants can be grouped into two classes (Class I and Class II) on the basis of cross resistance to other mitochondrial drugs (118). Class I mutants show cross resistance to aurovertin, Dio-9, venturicidin, triethyltin, uncouplers and other mitochondrial drugs. In contrast, Class II mutants are specifically resistant to oligomycin and structurally related antibiotics and shows no cross resistance to venturicidin, triethyltin, DCCD or uncoupling agents (122). All the Class II mutants exhibit typical cytoplasmic inheritance and the resistance determinants are located on the mtDNA. Genetic analysis indicates that two loci (OLI and OLII) located on independent cistrons on mtDNA are involved (118). Biochemical studies (121, 123) on the Class II mutants revealed that the ATPase, ATP-Pi exchange and ADP

stimulated respiration in isolated mitochondria were all less sensitive to oligomycin. Class II triethyl tin mutants have also been shown to be cytoplasmic and map at the T locus (121). Decreased sensitivity to triethyl tin is found in the ATPase and ADP stimulated respiration of Class II mutants (121).

Mitochondrial venturicidin resistant mutants map at two loci, those exhibiting cross-resistance to oligomycin mapping at the OLIII locus which is closely linked to OLI, and those exhibiting cross-resistance to triethyl tin mapping at the VI locus (120). Venturicidin mutants mapping at VI are very similar to the triethyl tin mutants mapping at TI and it seems likely that TI and VI are identical. All the available evidence suggests that OLI, OLII and VI are located on separate cistrons (120).

The resistance phenomenon may be explained by a binding of oligomycin at three attachment points, modification of which arise by mutation of OLI, OLII and OLIII. Venturicidin is complexed by two attachment points, one of which it has in common with oligomycin, the other being equivalent to the triethyl tin binding site (117, 120, 121).

CHAPTER 2

2. THE EFFECTS OF TRIALKYL TIN COMPOUNDS ON ENERGY-LINKED FUNCTIONS OF BEEF HEART MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

2.1 INTRODUCTION

Trialkyl tin compounds have been shown to be potent inhibitors of mitochondrial oxidative phosphorylation, oligomycin sensitive ATPase (OS-ATPase) and some energy-linked reactions, e.g. ^{32}Pi-ATP exchange (135-138). The mechanism by which the organotin compounds cause inhibition of these energy-linked reactions is not known. However, Aldridge et al. have proposed that inhibition is brought about by anyone of/or a combination of the following three mechanisms [summarised in Fig. 2.1 (140)]:

1. binding to a component of the energy conservation system to produce an oligomycin-like response;
2. uncoupling, due to their ability to mediate the exchange of halide for hydroxyl ions across membranes; and
3. uncoupling, due to induced gross swelling of the mitochondria.

Support for an oligomycin-like action of trialkyl tin compounds is obtained from the demonstration by Dawson and Selwyn (141) and Cain (142), that tributyl tin chloride and dibutyl-chloromethyl tin chloride (DBCT) block ADP stimulated respiration in mitochondria and lower the proton permeability of F_1-ATPase depleted ("stripped") submitochondrial particles (141). Studies on the binding of trialkyl tin compounds to submitochondrial particles of rat liver (143), beef heart (141) and yeast (145) have indicated the presence of one high affinity and one low affinity binding site for trialkyl tins. Aldridge et al. (143) have reported that the dissociation constant of the high affinity site of triethyl tin ($k_D \sim 10^{-8} M$) was similar to the concentration required for the inhibition of

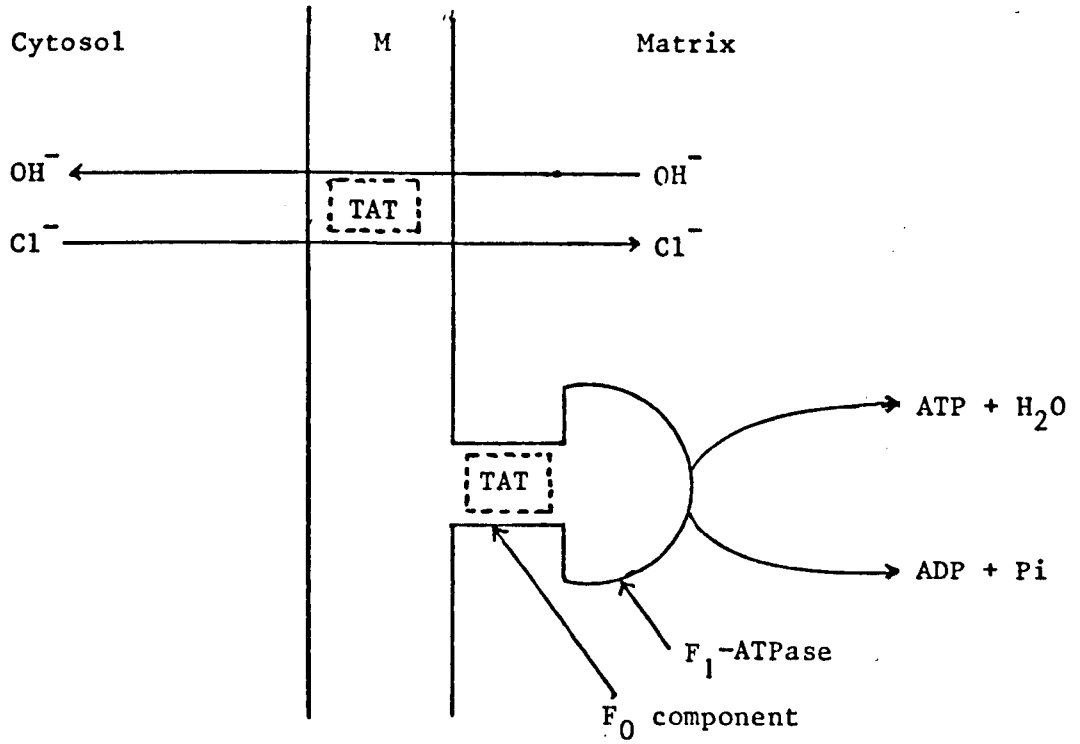


Fig. 2.1 Two mechanism by which trialkyl tins may inhibit oxidative phosphorylation. An oligomycin type inhibition; direct binding of trialkyl tin (TAT) to F_1F_0 ATPase complex; and uncoupler type action; catalysis of OH^-/Cl^- exchange across the coupling membrane; M, (140).

oxidative phosphorylation. Biochemical genetic studies by Griffiths and Lancashire (121) and binding studies by Cain et al. (144) have indicated that the high affinity binding sites of trialkyl tins were localized in the F_1F_0 ATPase complex. However, genetic studies on trialkyl tin resistant mutants of yeast by Griffiths et al. (117) and extraction of the 'trialkyl tin-X' complex (X = tin binding component) by Cain (142) (see Chapter 3 of this thesis), have shown that the high affinity sites are not localized on any of the protein components of the ATP synthesising system. These findings make it difficult to relate the mechanism of trialkyl tin inhibition to that of oligomycin or dicyclohexylcarbodiimide.

Trialkyl tins have also been proposed by Stockdale et al. (139, 145) to act as uncouplers of (see Appendix I) of oxidative

phosphorylation. These authors showed that trialkyl tins catalyse an anion/hydroxyl (Cl^-/OH^-) exchange across the inner membrane of mitochondria and chloroplasts, which results in the breakdown of the proton gradient responsible for driving ATP synthesis. An uncoupler mode of action has also been proposed by Selwyn et al. (146, 147) who have shown that trialkyl tins facilitate the swelling of mitochondria in Cl^- containing media. However, it is not known if the Cl^-/OH^- exchange system in mitochondria represents a specific property of the trialkyl tin compounds, or whether it is the result of interaction with specific sites in the mitochondrial membrane (e.g. components of the F_1F_0 ATPase complex).

Although an uncoupling mode of action might be used to explain trialkyl tin inhibition of oxidative phosphorylation, it cannot be used to explain the inhibition of mitochondrial membrane ATPase activity (145-147). It is thus, clearly important to establish the molecular basis for trialkyl tin inhibition of energy-linked reactions. Two approaches have been made:

(a) a study of the chemical specificity of trialkyl tins for the few proteins to which they bind (148), and

(b) the use of dibutylchloromethyl tin chloride (DBCT) as a covalent inhibitor of mitochondrial ATP synthase, followed by the isolation of the component(s) to which it is attached (142, 149).

The 'simple' chemical nature of trialkyl tin compounds, combined with their high biological activity and limited chemical reactivity, in addition to the fact that they appear to specifically inhibit oxidative phosphorylation and energy-linked reactions, make trialkyl tins useful probes in the investigation of the molecular mechanism of these reactions.

The present chapter describes the inhibitory properties of a number of trialkyl tin compounds; including dibutylchloromethyl tin chloride (DBCT) and a penta-coordinate tin compound {2-[(dimethylamino)methyl]phenyl}diethyl tin bromide (Ve_{2283}) on mitochondrial energy-linked reactions. In contrast to other trialkyl tin compounds, DBCT has been proposed to act as a specific covalent inhibitor of OS-ATPase activity and oxidative phosphorylation (150). Preliminary results showing that the OS-ATPase activity was 6-10 times more sensitive to all trialkyl tin compounds than oxidative phosphorylation was investigated more thoroughly using DBCT.

2.2 MATERIALS

All chemicals were of AnalaR or similar grade. Organic solvents of AnalaR grade were redistilled before use. Trialkyl tin chlorides and dicyclohexylcarbodiimide (DCCD) were obtained from BDH Chemicals Ltd., Poole, Dorset. Dibutylchloromethyl tin chloride was synthesised in this laboratory by Dr. D. E. Griffiths. {2-[(dimethylamino)methyl]phenyl}diethyl tin bromide (Ve_{2283}) and 4,5,6,7-tetrachloro-2-trifluoro-methylbenzimidazole (TTFB), were gifts from Professor B. Beechey, Shell Research Ltd., Sittingbourne, Kent. Oligomycin, valinomycin, gramicidin D, ATP, ADP hexokinase, glucose, rotenone, carbonyl-cyanide-m-chlorophenyl hydrazone (CCCP), dihydrolipoic acid, α-lipoic acid, glutathione 2,3-dimercaptopropanol, dithiothreitol and β-mercaptoethanol were obtained from Sigma Chemical Company. Antimycin A was obtained from Calbiochem, San Diego, California.

2.3 METHODS

Beef heart mitochondria and submitochondrial particles were prepared by the method of Low and Vallin (153). Freshly excised bovine hearts were brought on ice from a nearby slaughter house; all subsequent operations, preparation of submitochondrial particles and enzymes, were carried out at 4°C unless otherwise stated. Fat, connective tissue and ligaments were carefully trimmed from the muscle tissue which was then cut up into 3 cm cubes and passed through a meat grinder. The resulting minced tissue was resuspended in 2 volumes ice cold 0.25 M sucrose, the pH adjusted to pH 7.0-7.5 by addition of 1 M Tris base, and the suspension homogenised in a Waring tissue blender for 40 seconds at maximum speed. The suspension was then pH'd to a stable pH 7.0-7.5 by the addition of 1 M Tris base with rapid stirring; care being taken at this stage to ensure that the pH is stabilised and is not above 7.5. The suspension was then centrifuged at 2,000 r.p.m. in a Mistral 6 L centrifuge 4 x 1.25 L rotor for 30 minutes at 4°C. The supernatant was passed through 4 layers of muslin and the pH adjusted to 7.5 with 1 M Tris base before being centrifuged at 10,000 r.p.m. for 20 minutes in a Sorvall RC-2B centrifuge G.S.A. rotor, pre-cooled to 4°C. The supernatant from this spin was carefully removed by aspiration, as was the top light layer of the pellet. Any fat lining the centrifuge bottle was then carefully removed by wiping with a tissue. The dark brown mitochondrial pellet was then resuspended and homogenised in a glass homogeniser fitted with a teflon pestle in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5, diluted to 0.5-1.0 litres in the same buffer and recentrifuged at 10,000 r.p.m. for 20 minutes in a Sorvall RC-2B centrifuge G.S.A. rotor. The

supernatant was removed as previously and the pellet resuspended in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5 buffer and recentrifuged as above. The pellet from this centrifugation was resuspended in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 5 mM $MgCl_2$ and recentrifuged as above. The mitochondrial pellet was resuspended to 40 mg protein/ml in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA, 5 mM $MgCl_2$; 5mM succinate 2 mM ATP and the mitochondrial suspension either frozen immediately or kept at 4°C as required.

Submitochondrial particles were prepared by sonication of mitochondria. Frozen mitochondria were thawed rapidly and diluted to approximately 10 mg/ml protein concentration and sonicated for 1 minute (4 bursts of 15 seconds interspersed by 30 second gaps) in an MSE 60 W sonicator at maximum amplitude. The suspension was centrifuged at 15,000 r.p.m. for 15 minutes in a Sorvall RC-2B centrifuge 5534 rotor. The supernatant was then centrifuged at 100,000 g for 30 minutes in a Beckman L2-50 centrifuge, 40, 50 Ti or 65 rotor. The pellet from this centrifugation was resuspended in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA and recentrifuged as above. The submitochondrial pellet was resuspended in the same buffer to a final protein concentration of $\sim$ 20 mg/ml.

Electron transport particles (ETP_H) were prepared by the same method as submitochondrial particles except that the sonication buffer contained 2 mM sodium succinate; 2 mM ATP and 5 mM $MgCl_2$.

Complex V ATPase (F_1F_0 ATPase) was prepared by Hatefi and co-workers (151), using bovine heart submitochondrial particles (SMP's) as a starting material. SMP's were pelleted and resuspended in 0.66 M sucrose; 50 mM Tris-Cl, pH 8.0; 1 mM histidine to a protein concentration of 23 mg/ml. Potassium deoxycholate was added to a final

concentration of 0.3 mg/mg protein from a stock solution 10% w/v, pH 9.0. Potassium chloride was then added to 12 g/l and allowed to dissolve at 4° C with stirring. The suspension was then centrifuged at 30,000 r.p.m. for 30 minutes in a Beckman 30 rotor. The precipitate was discarded while the reddish supernatant was diluted with 0.25 volumes of double distilled water and recentrifuged at 30,000 r.p.m. for 40 minutes in the No. 30 rotor. The supernatant from this step was dialysed against 10-20 volumes 10 mM Tris-Cl pH 8.0 for 3 hours at 4° C. The dialysate was centrifuged for 90 minutes at 30,000 r.p.m. in the No. 30 rotor. The upper 2/3 of the supernatant from this step was removed from the loosely packed pellet, frozen in liquid nitrogen and stored until used. This supernatant was then passed through a Sephadex G25 column pre-equilibrated and eluted with 10 mM Tris-acetate, pH 7.5 at 100-130 ml per column of size 10 x 22 cm at 4° C. The pink column eluant was taken to 42% saturation with neutral saturated $(NH_4)_2SO_4$ at 4° C and centrifuged for 15 minutes at 70,000 g. The pellet from this step was resuspended in 0.25 M sucrose; 10 mM Tris-acetate, pH 8.0 at a concentration of 15-20 mg/ml protein. Potassium cholate was then added to 0.35-0.38 mg cholate/mg protein (from a 20% w/v stock solution pH 7.9) followed by $(NH_4)_2SO_4$ to 25% saturation. The suspension was then centrifuged after 10 minutes stirring at 4° C at 40,000 r.p.m. for 25 minutes in a No. 40 rotor. The precipitate was discarded and the supernatant taken to 42% saturation with saturated $(NH_4)_2SO_4$ at 4° C. The suspension was centrifuged as before and the pellet collected, redissolved to ∿ 20 mg/ml in 0.60 M sucrose; 50 mM Tris-Cl; pH 8.0; 1 mM histidine, frozen in liquid nitrogen and stored until used. This is complex V.

F_1 ATPase was prepared essentially as described by

Tyler and Webb (152), using bovine heart submitochondrial particles as starting material. Submitochondrial particles suspended at 10 mg protein/ml in buffer contain 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA; and 4 mM ATP; (STE-ATP buffer), were vortex-mixed rigorously on a Whirlimixer, and 0.5 vol. of chloroform added rapidly dropwise. When the chloroform addition was complete, vortex-mixing was continued for a further 20 seconds. The chloroform/water emulsion was broken by centrifugation in a bench centrifuge for 2 minutes. The upper aqueous layer was removed, diluted 1:1 with the initial buffer and centrifuged at 100,000 g for 30 minutes in Beckman L2-50 centrifuge 65 rotor. The supernatant containing the F_1 ATPase was stored in a water bath at 25°C. The ATPase was purified by $(NH_4)_2SO_4$ fractionation. All procedures were carried out at 25° C. Solid $(NH_4)_2SO_4$ (0.39 g/ml) was added slowly to a stirred sample of the supernatant. When all the $(NH_4)_2SO_4$ had been added and dissolved, the sample was left for 15 minutes and then recentrifuged at 12,000 g for 10 minutes. The supernatant was discarded and the white sediment redissolved in a volume of STE-ATP solution equal to ¼ vol. of the supernatant. The solution was left for 15 minutes and centrifuged for 4 minutes in an Eppendorf Microcentrifuge 3200 to remove a small amount of insoluble material. This supernatant contains the F_1 ATPase, which can be stored at 2° C as the $(NH_4)_2SO_4$ precipitate.

Oxidative phosphorylation: Oxidative phosphorylation was assayed in a glucose-hexokinase trap system. Assays contain 1.0-2.0 mg enzyme in 1.0 ml of 0.25 M sucrose; 20 mM glucose; 20 mM Tris-Cl, pH 7.4; 2 mM $MgCl_2$; 0.5 mM EDTA; 5.0 mM K_2HPO_4; 2 mM ADP and 5 units of hexokinase (usually either Sigma type F-300 or C-300. Enzymes were preincubated with any inhibitors, e.g. rotenone, antimycin A, oligomycin, uncouplers, etc., for 5 minutes prior to initiation of

assay in a wide-based phosphorylation tube in a shaking, heated water bath at 30° C. Assays were usually run for 20-30 minutes prior to termination. Assays were usually terminated by taking 0.5 ml aliquots into 0.2 ml 10% trichloroacetic acid (TCA) or 0.2 ml 1 M perchloric acid (PCA). Coagulated protein was removed by centrifugation in a bench centrifuge. Oxidative phosphorylation was measured either as the disappearance of phosphate from the medium or as the appearance of glucose-6-phosphate.

Estimation of glucose-6-phosphate: ATP can act as a phsophate donor to glucose in the presence of the enzyme hexokinase producing ADP and glucose-6-phosphate:

$$\text{ATP} + \text{D-glucose} \xrightarrow{\text{hexokinase}} \text{D-glucose-6-phosphate} + \text{ADP}$$

This reaction is used as the basis for the assay of ATP produced by oxidative phosphorylation. ATP is used by a glucose-hexokinase trap system to produce glucose-6-phosphate; the ADP released by this reaction can be rephosphorylated by the ATP synthase system to produce ATP. This allows the ADP concentration of the assay to be kept low as ADP can recycle until the free phosphate concentration of the assay becomes limiting.

After oxidative phosphorylation was terminated by taking 0.5 ml aliquot from the glucose-hexokinase trap system in 0.2 ml 1 M P.C.A., coagulated protein was removed by centrifugation. 0.5 ml aliquots from the supernatant of this step was taken into a tube containing enough 6 M KOH to neutralise it (75 µl). The tubes were kept at 0° C for 20 minutes to ensure complete precipitation of $KClO_4$ which was then removed by centrifugation. A 0.1 ml supernatant aliquot was taken into 1.9 ml 0.5 M Tris-Cl, pH 7.5, containing 0.5 µmoles $NADP^+$ and 2-3 units glucose-6-phosphate dehydrogenase which converts glucose-6-phosphate to glucono-δ-lactone-6-phosphate

with the concomitant reduction of $NADP^+$ to NADPH. NADPH was estimated from its absorption at 340 nm:

NADPH E_{mM} = 6.22 $cm^2/\mu mole$

Estimation of inorganic phosphate: Phosphate was determined by the method of Fiske and SubbaRow (156). This method is based on the formation of a phsophate molybdate complex which can be assayed spectrophotometrically. Phosphate will, in acid conditions, form a phosphomolybdous complex with ammonium molybdate. This can be reduced by an appropriate reducing agent (1-amino-2-naphthol-4-sulphonic acid) to form a phosphomolybdic complex which has a characteristic blue colour that can be assayed spectrophotometrically.

Assay Procedure: Inorganic phosphate (10-500 nmoles) in 0.5 ml water is added to an assay tube containing 1.5 ml water and 0.3 ml 2.5% ammonium molybdate in 5 N H_2SO_4. The contents are mixed on a vortex mixer and 0.2 ml of a 0.2% 1-amino-2-naphthol-4-sulphonic acid; 12% $NaHSO_3$; 2.4% $Na_2SO_3.7H_2O$ solution (ANSA reagent) is added. The tube is vortex mixed and allowed to stand for 20 minutes at room temperature after which time the optical density at 691 nm is read against a blank containing water, molybdate reagent and ANSA reagent. The A_{691} values obtained can be used to construct a standard curve for phosphate. Free phosphate concentration in assays, e.g. ATPase assays can be obtained by taking aliquots through the procedure above and comparing the A_{691} values to the standard curve.

ATPase assay: ATPase activity was determined by assaying phosphate released by the hydrolysis of ATP. Assays containing 100-200 µg mitochondrial protein or 10-20 µg purified ATPase protein were incubated in 1 ml 50 mM Tris-Cl, pH 8.5; 5 mM $MgCl_2$; 1 mM EDTA (assays were carried out at pH 8.5 unless specified otherwise) for 5 minutes with effectors and inhibitors, if any were present in

the assay at 30° C prior to initiation of the assay with 5 µmoles ATP. Assays were terminated after 5-15 minutes by addition of 0.5 ml 10% TCA. Coagulated protein was removed by centrifugation in a bench centrifuge, and a 0.5 ml aliquot of the supernatant was taken into an assay to determine inorganic phosphate (Pi).

Protein determination: Mitochondrial protein was estimated by the biuret method of Gornall et al. (154). Purified enzyme protein was estimated by the Folin-Lowry method described by Lowry et al. (155).

Energy linked transhydrogenase assay: Assays of ATP and succinate driven transhydrogenase were as described by Beechey et al. (157). 1.0-1.2 mg submitochondrial particles were incubated in 3.0 ml 50 mM Tris-Cl, pH 8.0 buffer containing 5.0 mM $MgCl_2$, 0.25 M sucrose; 66 µM NAD^+, 330 µM $NADP^+$, 10 mM ethanol, 300 µg of yeast ethanol dehydrogenase and 1 µM KCN for 3 minutes at 30° C with or without inhibitor. ATP, 5 µmol or succinate, 50 µmol was then added to initiate the reaction. Reduction of $NADP^+$ to NADPH was followed at 340 nm in a Unicam S.P. 1800 spectrophotometer. 1 µg rotenone and 1 µg antimycin A were also added to the ATP driven reaction.

Preparation of dihydrolipoate solutions: Unless otherwise stated, dihydrolipoic acid solutions were normally prepared as follows. Dihydrolipoic acid (obtained from Sigma Chemicals) was weighed out into a de-ionised glass or borosilicate vial: enough 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA was added to give 0.4 M (or 0.2 M) solution. Tris base (1 M) was then added until dissolution of dihydrolipoate was obtained; the final concentration of the solution was adjusted to 0.2 M (or 0.1 M) by addition of the requisite volume of 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA buffer. The

final pH of these solutions was usually 7.5-7.8. The concentration of dihydrolipoic acid was usually checked, using the method of Ellman (158) which assay the presence of thiols. Dihydrolipoic acid solutions are usually made up fresh (when required), they were never stored or kept for any length of time. Solutions of 2,3 dimercaptopropanol were usually made up in methanol.

2.4 RESULTS

The results presented on Figs. 2.2 and 2.3 show that most of the organotins investigated were potent inhibitors of the OS-ATPase activity of mitochondria and submitochondrial particles. A comparison of the I_{50} values of the different organotin compounds for the OS-ATPase of mitochondria and submitochondrial particles, show that the inhibitory potency of DBCT ($I_{50} \sim 1.0 \pm 0.15$ nmol/mg protein) was similar to that of the other trialkyl tin compounds, both in mitochondria and submitochondrial particles (Table 2.1). In contrast Ve_{2283} ($I_{50} \sim 0.1$ nmol/mg protein) was found to be 10 times more potent than DBCT, while trimethyl tin chloride was almost ineffective as an inhibitor. Dibutyl tin dichloride (DBT) was also found to be a good inhibitor of the OS-ATPase activity of mitochondria and submitochondrial particles. The latter finding is in agreement with that reported by Cain et al. (159) but disagrees with the proposal of Aldridge that dialkyl tins were effective inhibitors, only of the α-keto acid dehydrogenase of the TCA cycle (160, 161).

The data presented in Table 2.2, shows that, like oligomycin DBCT, Ve_{2283} and the other trialkyl tin compounds were potent inhibitors of the OS-ATPase activity in mitochondria, submitochondrial particles and complex V [prepared by the method of

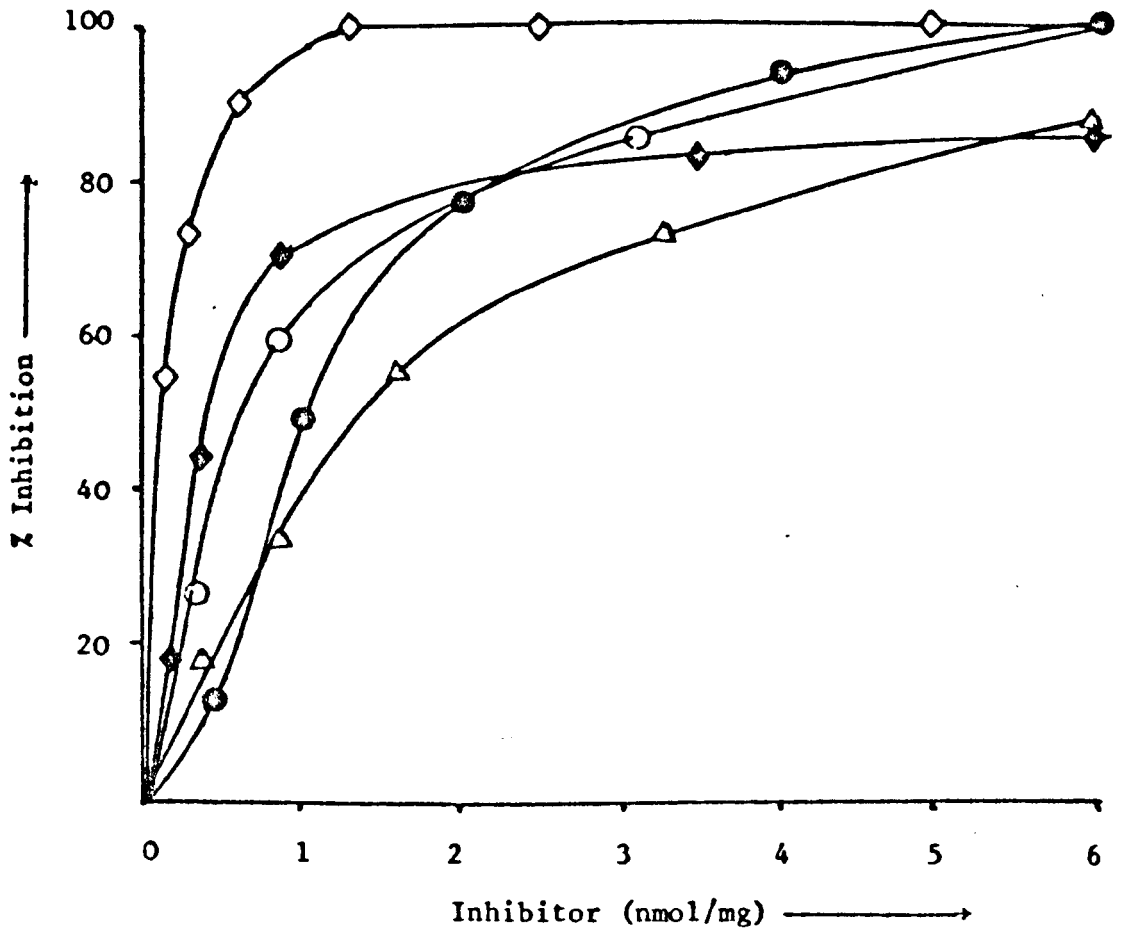


Fig. 2.2 Inhibition of OS-ATPase activity in beef heart mitochondria by trialkyl tins

Mitochondria suspended at 10 mg protein/ml in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA, was preincubated with varying concentrations of trialkyl tins at 4° C for 30 minutes and the ATPase activity assayed as described in Methods. The specific activity of the starting mitochondria was 0.8 µmoles ATP hydrolysed/min/mg. Experimental data have been expressed in the figure as a per cent inhibition of this original value. (◊), Ve_{2283}; (◆), tripropyl tin chloride; (⊙), DBCT; (○), tributyl tin chloride; (△), dibutyl tin dichloride.

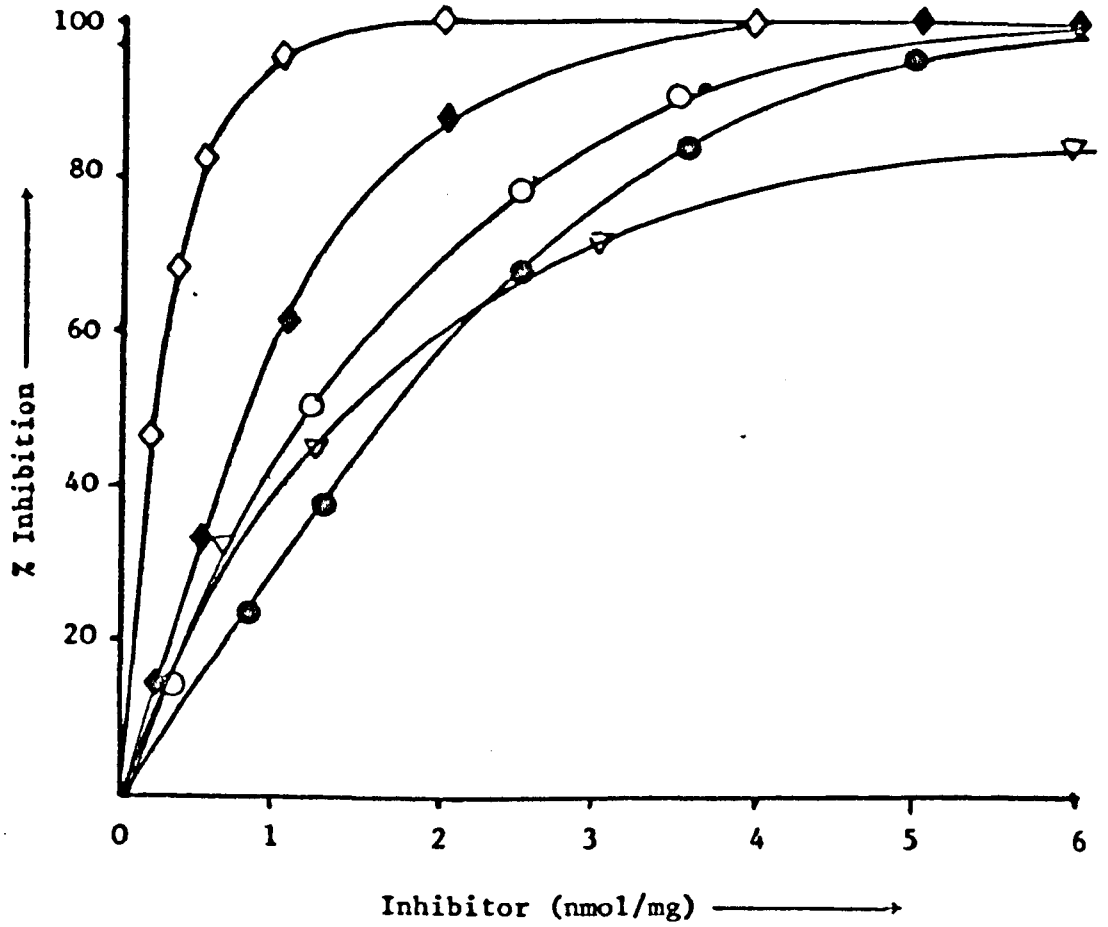


Fig. 2.3 Inhibition of OS-ATPase activity in beef heart submitochondrial particles by trialkyl tins

Submitochondrial particles suspended at 10 mg protein/ml in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA, were preincubated with varying concentrations of trialkyl tins at 4° C for 30 minutes and the ATPase activity assayed as described in Methods. The specific activity of the starting submitochondrial particles was 2.4 µmoles ATP hydrolysed/min/mg. Experimental data have been expressed in the figure as a per cent inhibition of this original value. (◇), Ve_{2283}; (◆), DBCT; (○), tributyl tin chloride; (◎), tripropyl tin chloride; (▽), dibutyl tin dichloride.

Table 2.1 Trialkyl tin inhibition of OS-ATPase activity in mitochondria and submitochondrial particles

Trialkyl tin	Mitochondria I_{50} (nmoles/mg protein)	SMP I_{50} (nmoles/mg protein)
Trimethyl tin chloride	-	-
Triethyl tin chloride	2.4 ± 0.1	2.8 ± 0.2
Tripropyl tin chloride	0.5 ± 0.1	1.8 ± 0.3
Tributyl tin chloride	0.8 ± 0.2	1.2 ± 0.15
Dibutylchloromethyl tin chloride (DBCT)	0.8 ± 0.1	0.8 ± 0.2
Triphenyl tin chloride	1.6 ± 0.3	1.2 ± 0.2
Tetraethyl tin	-	-
Ve_{2283}	0.10	1.2 ± 0.05
Dibutyl tin dichloride	2.00 ± 0.2	1.8 ± 0.1

ATPase activity was assayed as in Methods. Conditions of incubation with trialkyl tins were as described in the legends of Fig. 2.2 and Fig. 2.3. Each value presented is the mean ± standard deviation (s.d.) of five duplicates. Specific ATPase activities were for mitochondria 0.85 µmol/min/mg; SMP's, 2.4 µmol/min/mg.

Hatefi et al. (151)], but they did not affect the F_1 ATPase activity. The I_{50} value was found to increase from 0.8 ± 0.1 nmol/mg protein in mitochondria to 4.8 ± 0.2 nmol/mg protein in complex V when DBCT was the inhibitor. This represents a 5-6 fold increase in the I_{50} value for DBCT. In general, a 3-6 fold increase in the I_{50} value was found for the other trialkyl tins investigated (Table 2.2). This 3-6 fold increase in the I_{50} values might be due to an equivalent increase in the number of trialkyl tin binding sites/mg protein, caused by the 'effective' purification of the OS-ATPase complex. The finding that the F_1 ATPase is insensitive to inhibition by trialkyl tins is in agreement with the effect of trialkyl tins on

Table 2.2 Comparison of the I_{50} values of trialkyl tins of various ATPase preparations

Trialkyl tin	I_{50} values of ATPase preparations nmoles/mg protein			
	Mitochondria	SMP	Complex V	F_1-ATPase
Triethyl tin chloride	2.4	2.8	6.3	-
Tributyl tin chloride	0.8	1.2	5.4	-
Tripropyl tin chloride	0.5	1.8	5.8	-
DBCT	0.8	0.8	4.8	-
Ve_{2283}	0.1	0.2	0.6	-
Triphenyl tin chloride	1.6	1.2	4.7	-
Dibutyl tin dichloride	2.0	1.8	-	-

All conditions of inhibition and assay of OS-ATPase activity were identical to those described in the legend of Table 2.1. Each value presented is the mean of four duplicates. The specific ATPase activities of the starting mitochondria was 0.8 µmol/min/mg; submitochondrial particles, 2.4 µmol/min/mg; complex V, 14.8 µmol/min/mg; F_1-ATPase, 38.6 µmol/min/mg.

F_1-ATPase reported by Cain *et al.* (142) and others (139-143). The obvious interpretation of these results, is that the F_0 component of the OS-ATPase is necessary for the inhibition of the OS-ATPase activity by trialkyl tins; that is, trialkyl tins are specific OS-ATPase inhibitors.

Cain *et al.* (142, 159) found that triethyl tin chloride and dibutyl tin dichloride (DBT) like oligomycin, inhibited ADP stimulated respiration in beef heart mitochondria utilizing pyruvate/malate or succinate as substrate, but did not affect uncoupler stimulated respiration. These results suggest a direct action of the trialkyl tins on the ATP-synthase complex. This suggestion was examined by studying the effect of DBCT, Ve_{2283} and

other trialkyl tins on ATP synthesis in beef heart mitochondria and submitochondrial particles. Fig. 2.4 and Fig. 2.5 respectively show that succinate driven ATP synthesis in mitochondria and submitochondrial particles is inhibited by all the trialkyl tins examined. From the results presented in Table 2.3 (and on Fig. 2.4 and Fig. 2.5), it can be seen that Ve_{2283} with an I_{50} value of 1.0 ± 0.1 nmol/mg protein and causing maximal inhibition of oxidative phosphorylation at 2.0 ± 0.12 nmol/mg protein, is the most potent of the organotin compounds so far examined. DBCT with an I_{50} value of 5-7 nmol/mg protein and causing maximal inhibition at 15-20 nmol/mg protein, is similar in potency to most of the other trialkyl tins examined (Table 2.3). The data presented in Table 2.3 also shows that the I_{50} values of the trialkyl tin compounds are not significantly different for oxidative phosphorylation in mitochondria and submitochondrial particles. These results are in agreement with the findings of Cain et al. (142, 159), but contradicts with the findings of Aldridge (161), that 30 times the amount of trialkyl tin was needed to inhibit oxidative phosphorylation in mitochondria than was needed to inhibit oxidative phosphorylation in submitochondrial particles.

Preliminaty data, presented in Tables 2.2 and 2.3 and by Cain et al. (142, 159) and Aldridge (161), indicating differential sensitivity of the OS-ATPase activity and oxidative phosphorylation in mitochondria and submitochondrial particles to inhibition by trialkyl tins, was investigated more thoroughly using DBCT and tributyl tin chloride. The results presented on Figs. 2.6 and 2.7 show that the OS-ATPase activity in both mitochondria and submitochondrial particles was 85-90% inhibited at low concentration of DBCT (2-3 nmol/mg protein) which did not significantly affect oxidative phosphorylation

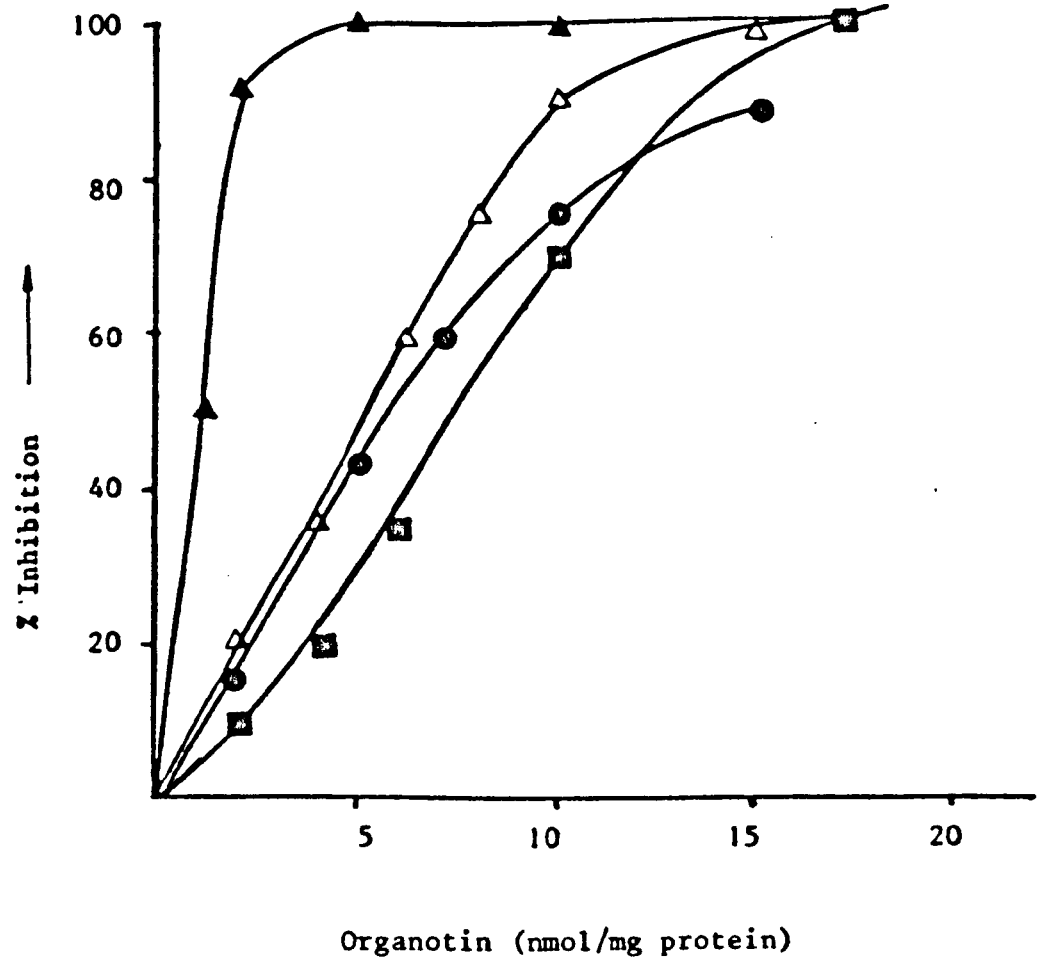


Fig. 2.4 Inhibition of succinate driven ATP synthesis in beef heart mitochondria by trialkyl tins

Mitochondria suspended at 10 mg protein/ml in 0.25 M sucrose; 10 mM tris-Cl, pH 7.5; 1 mM EDTA; buffer, were preincubated with varying concentrations of trialkyl tins at 4° C for 30 minutes. Aliquots (1 mg ≡ 100 µl) were then removed and assayed for succinate driven ATP synthetic activity as described in Methods. The specific ATP synthetic activity of the starting mitochondria was 130 nmol ATP synthesised/min/mg protein. Experimental data have been expressed in the figure as a per cent inhibition of this original value. Each data point is the average of duplicates. (▲), Ve_{2283}; (△), DBCT; (●), triphenyl tin chloride; (■), tributyl tin chloride.

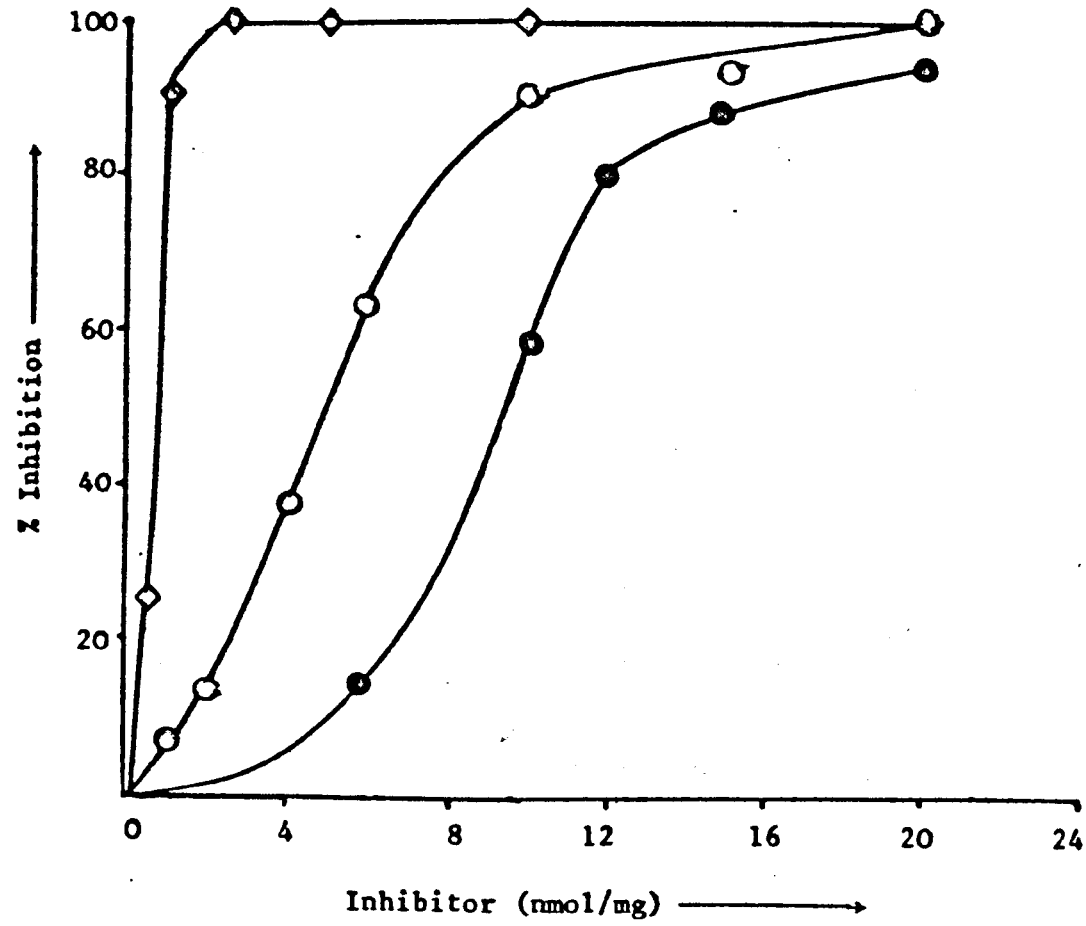


Fig. 2.5 Inhibition of succinate driven ATP synthesis in beef heart submitochondrial particles

Submitochondrial particles (SMP) suspended at 10 mg protein/ml in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, were preincubated with varying concentrations of trialkyl tin compounds at 4° C for 30 minutes and ATP synthetic activity assayed as described in Methods. The specific ATP synthetic activity of the starting SMP's was 90 nmol of ATP synthesised/min/mg. Experimental data have been expressed in the figure as a percent inhibition of this original value. (◇), Ve_{2283}; (○), DBCT; (●), tributyl tin chloride. Each data point is the average of 4 duplicates.

Table 2.3 Inhibition of succinate driven ATP synthesis in mitochondria and submitochondrial particles by trialkyl tins

Trialkyl tin	Mitochondria I_{50} (nmoles/mg protein)	Submitochondrial particles I_{50} (nmoles/mg protein)
Triethyl tin chloride	9.8	10.6
Tripropyl tin chloride	7.6	8.6
Tributyl tin chloride	7.8	10.0
DBCT	5.6	6.6
Ve_{2283}	1.2	1.0
Triphenyl tin chloride	5.8	6.6

All conditions of inhibition and assay of ATP synthetic activity were identical to those described in the legends of Fig. 2.4 and Fig. 2.5. Each value presented is the average of four duplicates. The specific ATP synthetic activity of mitochondria is 130 nmol ATP synthesised/min/mg and of submitochondrial particles is 90 nmol ATP synthesised/min/mg.

in submitochondrial particles, but cause 20-30% inhibition in mitochondria. Similar results were obtained with tributyl tin chloride (Figs. 2.6, 2.7). The ATPase and oxidative phosphorylative activities were assayed under (close to) identical conditions, except that hexokinase, ADP, and inorganic phosphate, were omitted from the ATPase assay buffer which contained 0.25 M sucrose; 5 mM $MgCl_2$; 20 mM Tris-Cl, pH 7.5; 20 mM succinate; 0.5 mM EDTA and 20 mM glucose. The residual 60-80% ATP synthetic activity of mitochondria and submitochondrial particles was sensitive to oligomycin, Ve_{2283} and the uncoupler 2,4-dinitrophenol (Table 2.4). These results indicate that the mode of inhibition of oxidative phosphorylation and ATP hydrolysis by DBCT (or tributyl tin chloride) might be different, or that the catalytic or inhibitor binding sites

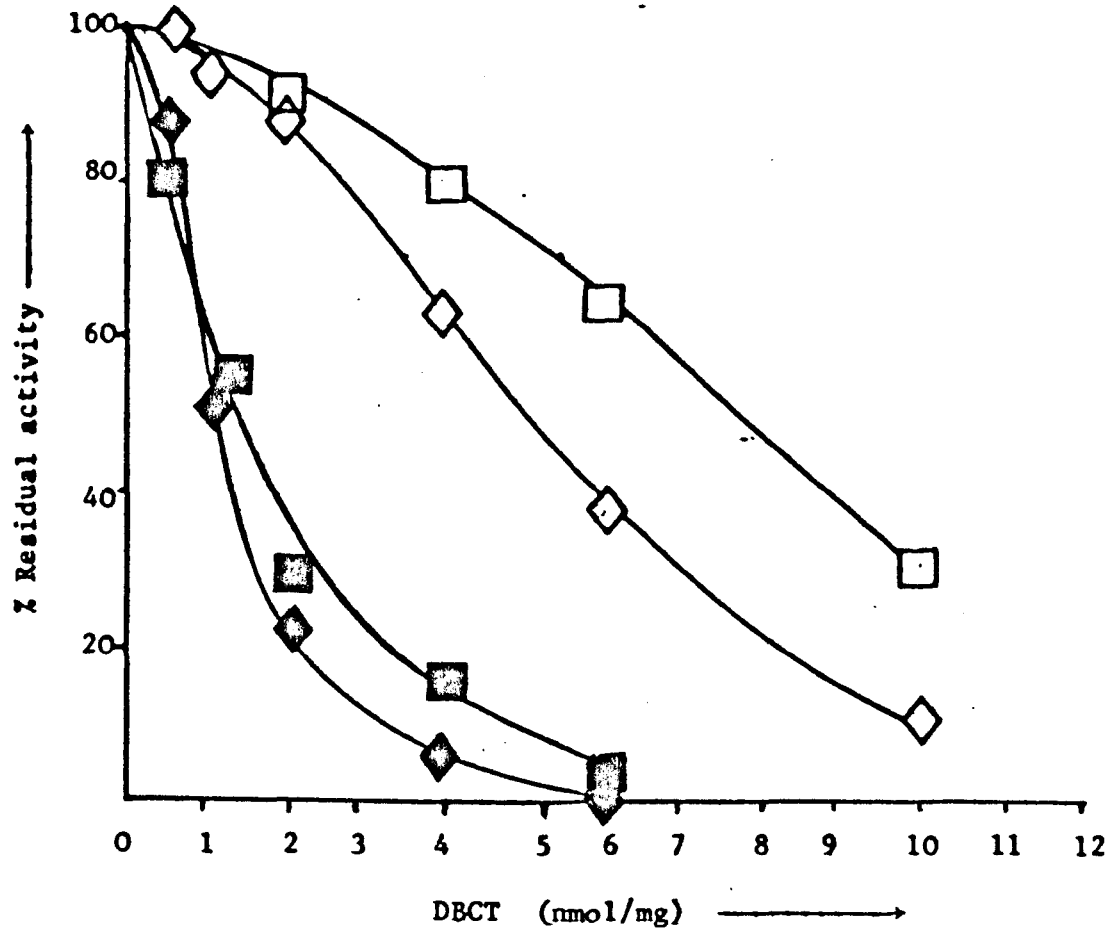


Fig. 2.6 Inhibition of succinate driven ATP synthesis and ATP hydrolysis in beef heart mitochondria by DBCT and tributyl tin chloride (TBT).

Mitochondria was preincubated with varying concentrations of DBCT or TBT, under the conditions described in Table 2.4. ATP synthesis and hydrolysis were assayed as in Table 2.4. The specific activities of the starting mitochondria were: ATPase, 0.5 µmol ATP hydrolysed/min/mg; ATP synthesis, 120 nmol ATP synthesised/min/mg. Experimental data were expressed in the figure as a per cent of these original values (◆), DBCT, (■), TBT inhibition of ATP hydrolysis; (◇), DBCT, (□), TBT inhibition of ATP synthesis. Each point is the average of the 4 duplicates.

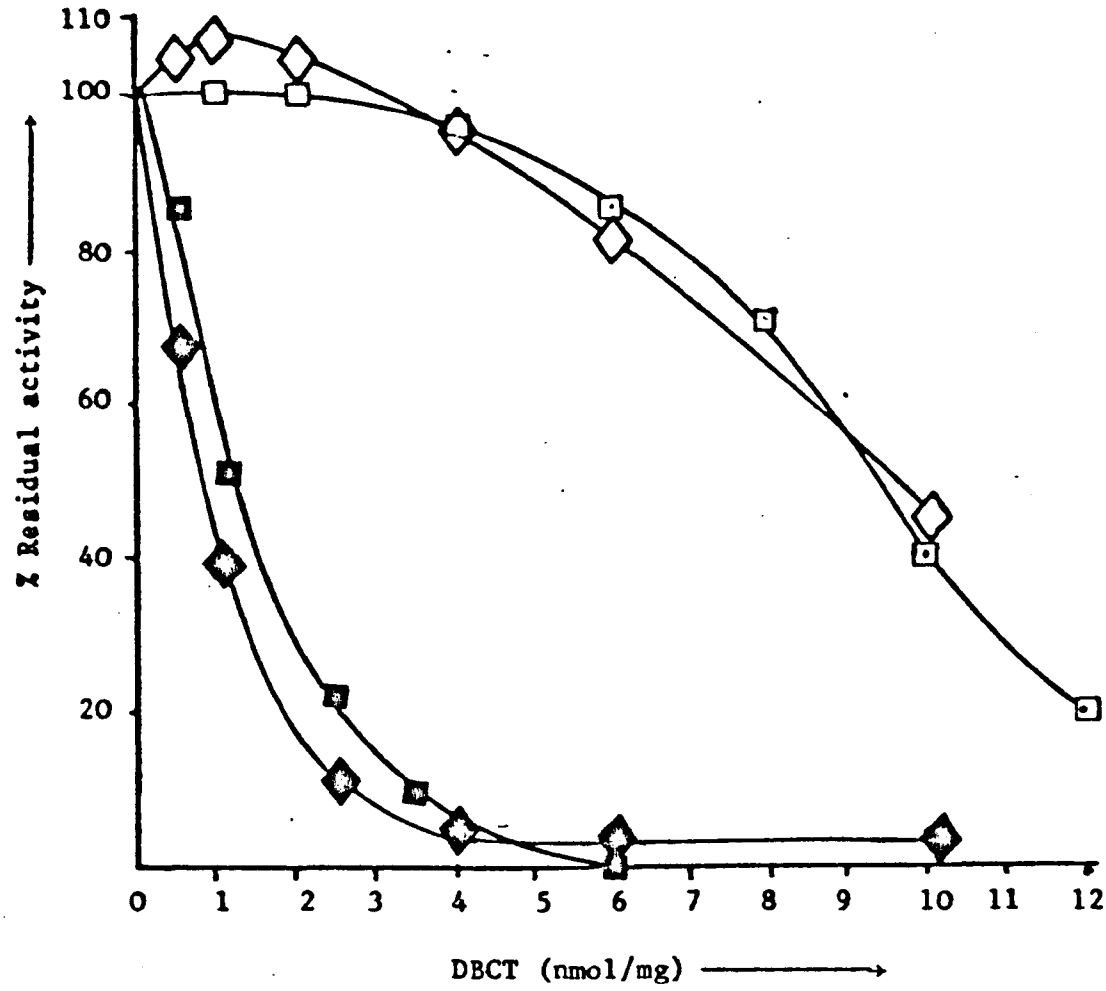


Fig. 2.7 Inhibition of succinate driven ATP synthesis and ATP hydrolysis in submitochondrial particles (SMP's) by DBCT and tributyl tin chloride

All conditions of inhibition and assay of ATP synthetic and ATP hydrolytic activities were as described in Fig. 2.6. The specific activities of the starting SMP's were: ATPase, 1.4 μmol ATP hydrolysed/min/mg; ATP synthesis, 100 nmol ATP synthesised/min/mg. Experimental data have been expressed in the figure as a percent of these original values. (◆), DBCT, (■) TBT inhibition of ATP hydrolysis; (◇), DBCT, (□), TBT inhibition of ATP synthesis. Data points are the average of 4 duplicates.

involved in ATP synthesis and ATP hydrolysis are different.

Table 2.4 Differential sensitivity of the ATP synthetic and ATP hydrolysing activities in mitochondria and submitochondrial particles to DBCT

Additions	ATPase µmol/min/mg	ATP synthesis nmol/min/mg
Mitochondria	0.5	120.0
Mitochondria + DBCT (3.3 nmol/mg)	0.0	75.0
Mitochondria + DBCT + DNP (2 µg)	0.0	0.0
Mitochondria + DBCT + Ve_{2283} (4 nmol)	0.0	0.0
Mitochondria + DBCT + Oligomycin (2 µg)	0.0	0.0
SMP	1.40	100.0
SMP + DBCT (3.3 nmol/mg)	0.10	95.0
SMP + DBCT + DNP (2 µg)	0.10	0.0
SMP + DBCT + Ve_{2283} (4 nmol)	0.0	0.0
SMP + DBCT + Oligomycin (2 µg)	0.0	0.0
		0.0

Mitochondria and submitochondrial particles suspended at 10 mg protein/ml in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA, were preincubated at 3.3 nmol/mg protein with DBCT at 4° C for 30 min. The ATPase activity was assayed as in Methods, in a 20 mM Tris-Cl buffer pH 7.5 containing: 20 mM succinate; 0.5 mM EDTA, 0.25 M sucrose 5 mM $MgCl_2$ and 20 mM glucose. Succinate driven ATP synthesis was assayed as described in Methods. Oligomycin, 2 µg; 2,4-dinitrophenol (DNP) 2 µg and Ve_{2283}, 3 nmoles were added as methanolic solutions. Experimental data have been expressed in the Table as specific activities of the system assayed. The data presented are the mean of four duplicates.

To gain more insight into the mode(s) of action of trialkyl tin compounds, the effect of DBCT, Ve_{2283} and other trialkyl tins on energy-linked NADH/NADP transhydrogenase was investigated. The results presented on Fig. 2.8 show that DBCT, Ve_{2283} and the other trialkyl tins

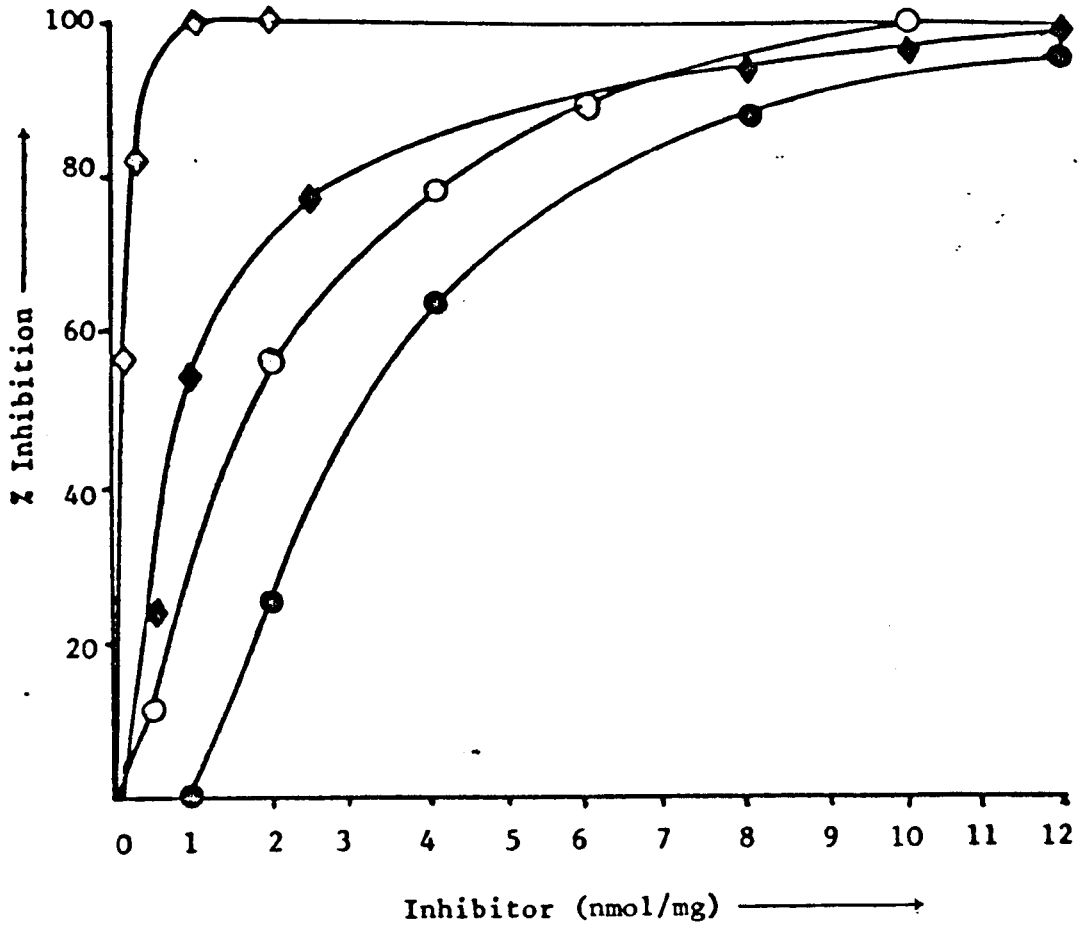


Fig. 2.8 Inhibition of ATP-driven transhydrogenase in beef heart submitochondrial particles by trialkyl tin compounds

The condition of inhibition with trialkyl tins were identical to those described in the legend of Table 2.5. ATP driven transhydrogenase was assayed as described in Methods. The specific transhydrogenase activity of the starting submitochondrial particles was 38.0 ± 3.6 nmol NADPH formed/min/mg. Experimental data have been expressed in the figure as a per cent inhibition of this original value. Data points are the average of duplicates. (◇), Ve_{2283}; (◆), triphenyl tin chloride; (○), DBCT; (●), dibutyl tin dichloride.

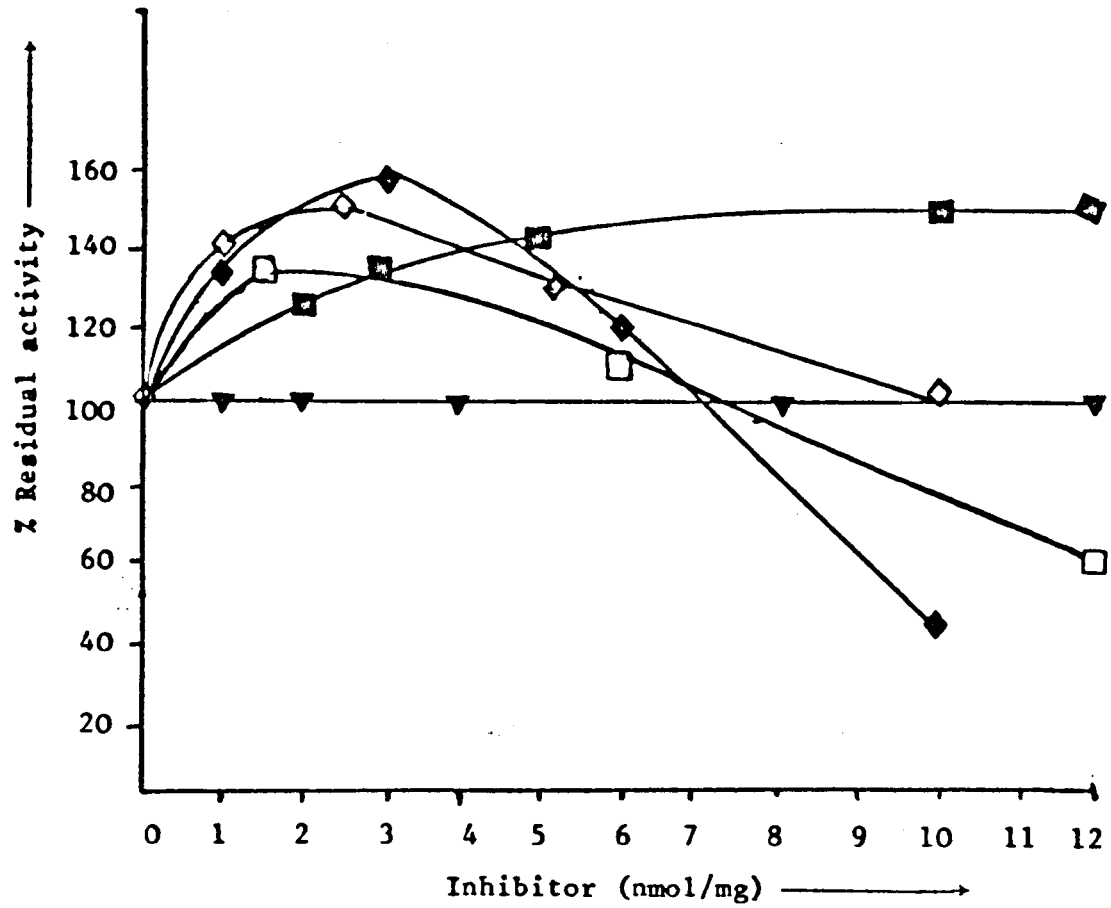


Fig. 2.9 The effect of trialkyl tin compounds on succinate driven transhydrogenase activity in beef heart submitochondrial particles

The condition of inhibition with trialkyl tins were identical to those described in the legend of Table 2.5. The specific transhydrogenase activity of the starting submitochondrial particles was 60 ± 5.0 nmol of NADPH formed/min/mg. Experimental data have been expressed in the figure as a percent of this original value. Each data point is the mean of duplicates. Transhydrogenase activity was measured as described in Methods. (◇), Ve2283; (■), oligomycin; (□), DBCT; (◆), triphenyl tin chloride; (▼), dibutyl tin chloride.

were potent inhibitors of ATP driven transhydrogenation. The I_{50} values of these compounds, presented in Table 2.5, show that the sensitivity of the ATP-driven reaction is similar to that obtained for the OS-ATPase activity in submitochondrial particles (Table 2.1). Similar I_{50} values have been reported by Cain (142) for the ATP driven succinate reduction of NAD^+ by submitochondrial particles (e.g. 0.9 nmol DBCT/mg protein), suggesting that the OS-ATPase plays an important role in these ATP-dependent reactions. In the case of succinate driven transhydrogenation (Fig. 2.9), all the organotins except dibutyl tin dichloride, were found to stimulate the succinate driven transhydrogenase reaction at low concentrations (1-4 nmol/mg protein). However, unlike oligomycin, higher concentrations (10-15 nmol/mg protein) of trialkyl tins were found to inhibit succinate driven transhydrogenation.

The results of the energy-linked reactions, indicate that the trialkyl tins studied have two possible modes of action:

1. an oligomycin like action responsible for the inhibition of OS-ATPase and ATP-driven energy linked reactions;
2. and an uncoupling action (cf. CCCP) responsible for the inhibition of succinate driven (respiratory dependent) transhydrogenation and oxidative phosphorylation.

2.5 REVERSAL OF ORGANOTIN INHIBITION WITH DITHIOLS

Cain et al. have shown that the normally irreversible inhibition of the OS-ATPase activity of beef heart mitochondria by DBCT and dibutyl tin dichloride, could be reversed by dihydrolipoic acid (159). Consequently, we have investigated the effect of a number of monothiols and dithiols on the inhibition of OS-ATPase, oxidative phosphorylation and ATP-driven transhydrogenation by a

Table 2.5 Trialkyl tin inhibition of ATP-driven transhydrogenase activity in submitochondrial particles

Trialkyl tin	I_{50} values nmol/mg protein
Triethyl tin chloride	2.6
Tripropyl tin chloride	1.8
Tributyl tin chloride	1.4
DBCT	1.2
Dibutyl tin dichloride	3.2
Triphenyl tin chloride	0.8
Ve_{2283}	0.2

ATP driven transhydrogenase activity was assayed as described in Methods. Submitochondrial particles suspended at 10 mg protein/ml in 0.25 M sucrose; 10 mM Tri-Cl, pH 7.5; 1 mM EDTA; were preincubated with varying concentrations or organotin compounds at 4° C for 30 min. 100 µl aliquots were removed and assayed for ATP-driven transhydrogenase activity. The specific activity of the starting submitochondrial particles was 38.0 nmol NADPH formed/min/mg. The values presented are the mean of four duplicates.

number of trialkyl tin compounds.

The inhibition of succinate driven oxidative phosphorylation by most of the trialkyl tin compounds examined, except Ve_{2283}, was found to be completely reversible by 2,3-dimercaptopropanol. In the case of DBCT inhibition of oxidative phosphorylation in mitochondria (Fig. 2.10) and submitochondrial particles (Fig. 2.11) 60 nmol/mg and 20 nmol/mg protein of 2,3-dimercaptopropanol respectively were found to give complete reversal. Dithiothreitol (DTT) and dihydrolipoic acid were found to be totally ineffective as reversal agents at such low concentrations (Table 2.6); 100 nmol/mg protein dithiothreitol or dihydrolipoic acid giving less than 10% reversal. However, dihydrolipoic acid and dithiothreitol were found

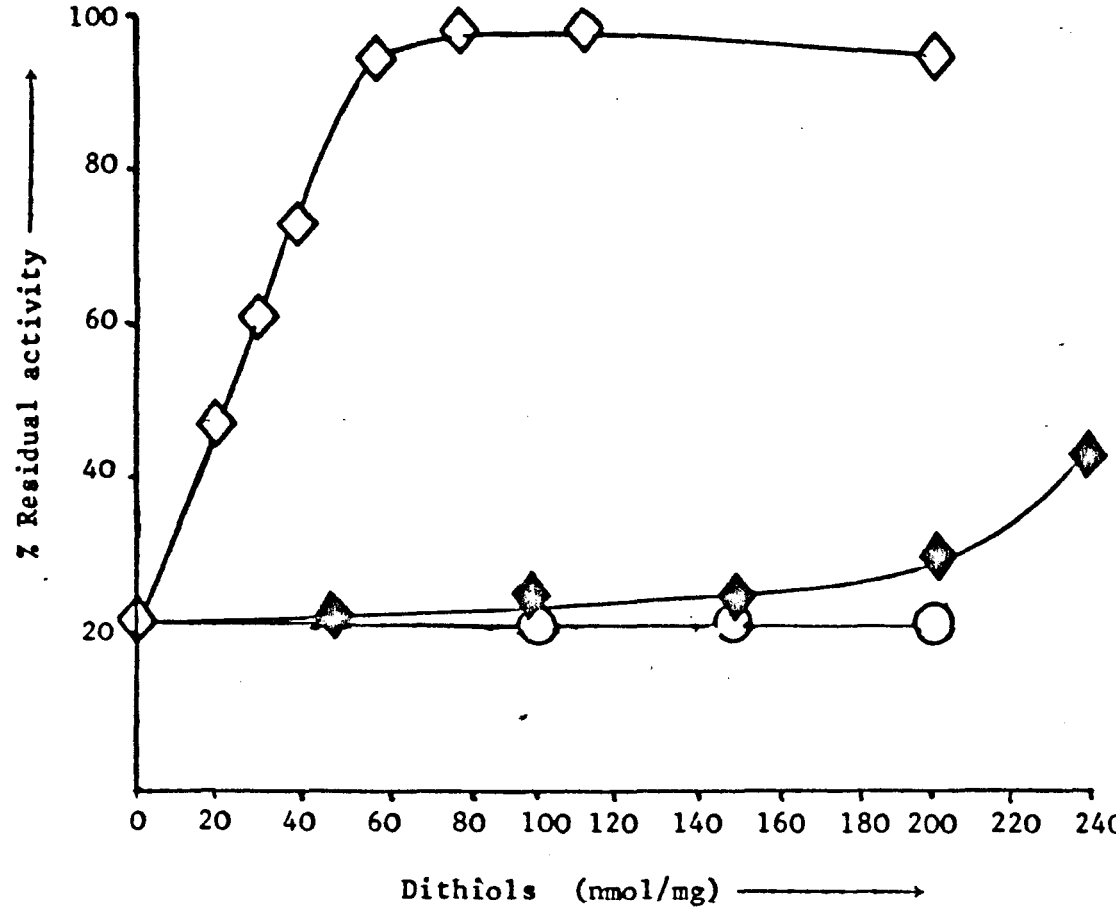


Fig. 2.10 Dithiol reversal of DBCT inhibition of succinate driven ATP-synthesis in beef heart mitochondria

Mitochondria suspended at 10 mg/ml were preincubated with DBCT (15 nmol/mg), treated with dithiols and the ATP synthetic activity assayed as described in Fig. 2.12. Specific ATP synthetic activity of starting mitochondria was 140 nmol ATP synthesised/min/mg. Data expressed in figure as a percent of this original value. Data points are the average of 4 duplicates. (◇), 2,3-dimercaptopropanol; (◆), dihydrolipoic acid; (○), dithiothreitol.

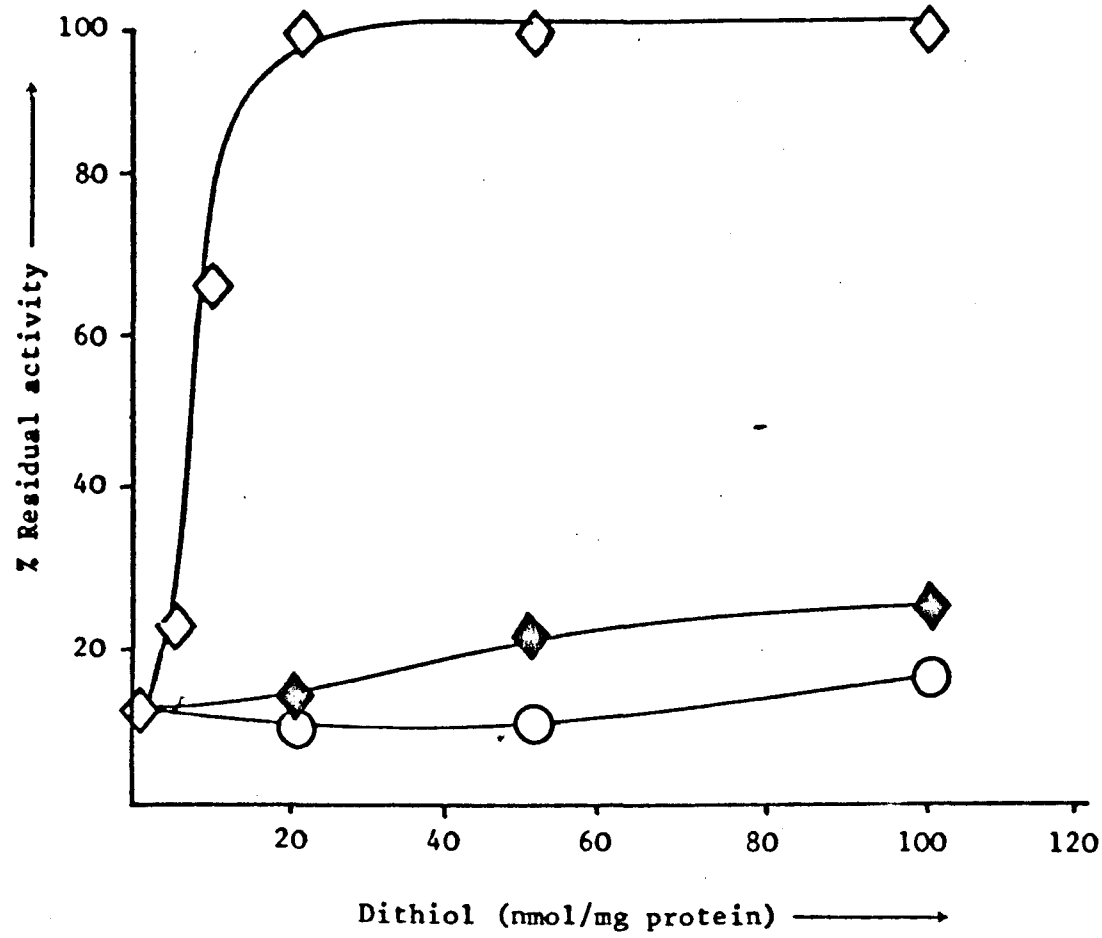


Fig. 2.11 Dithiol reversal of DBCT inhibition of succinate driven ATP synthesis in beef heart submitochondrial particles (SMP's)

DBCT-treated submitochondrial particles (20 nmoles DBCT/mg) suspended at 10 mg/ml were treated with dithiol as described in Fig. 2.10, and assayed for oxidative phosphorylation activity as described in Methods. Specific ATP synthesising activity of the starting SMP's was 94 nmol ATP synthesised/min/mg. Experimental data have been expressed in the figure as a percent of this original value. (◇), 2,3-dimercaptopropanol; (◆), dihydrolipoic acid; (○), dithiothreitol. Each data point is the average of 4 duplicates.

Table 2.6 Dithiol reversal of DBCT and DBT inhibition of oxidative phosphorylation in mitochondria

Additions	ATP Synthesised nmol/min/mg	% Activity
None	140	100.0
DBCT (15 nmol/mg)	23	16.4
DBCT + 2,3-dimercaptopropanol (50 nmol/mg)	135	96.5
DBCT + dihydrolipoic acid (50 nmol/mg)	25	17.9
DBCT + dithiothreitol (50 nmol/mg)	20	14.3
DBCT + mercaptoethanol (50 nmol/mg)	20	14.3
DBCT + Glutathione (50 nmol/mg)	20	14.3
DBT (30 nmol/mg)	25	17.9
DBT + 2,3-dimercaptopropanol (50 nmol/mg)	140	100
DBT + dihydrolipoic acid (50 nmol/mg)	135	96.5
DBT + dithiothreitol (50 nmol/mg)	130	92.9
DBT + mercaptoethanol (50 nmol/mg)	30	21.4
DBT + glutathione (50 nmol/mg)	30.0	21.4

Mitochondria suspended at 10 mg/ml in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA, were preincubated with 15 nmol DBCT/mg protein or 30 nmol DBT/mg protein at 4° C for 30 min. 100 µl (1 mg protein) aliquots were then placed in 1 ml phosphorylation buffer in a reaction tube (containing 2 µg retenone); 50 nmol of the thiol or dithiol was added to each tube and preincubated for 5 minutes at 30° C. Succinate driven oxidative phosphorylation was assayed as described in methods. Each value presented is the average of four duplicates.

to be much more effective at higher concentrations; 500 nmol/mg giving 48% and 28% reversal respectively. Triphenyl tin chloride and dibutyl tin dichloride inhibition of oxidative phosphorylation in mitochondria and submitochondrial particles were totally reversed by dithiothreitol, dihydrolipoic acid and 2,3-dimercaptopropanol at 50-100 nmol/mg. However, tributyl tin inhibition of oxidative phosphorylation could only be reversed 50% by 20 nmol 2,3-dimercaptopropanol/mg protein, and was not significantly reversed by dihydrolipoic acid or dithiothreitol.

No significant reversal of trialkyl tin inhibition of OS-ATPase activity in mitochondria or submitochondrial particles was obtained with the dithiols at the relatively low concentrations that (20-60 nmol/mg) caused reversal of oxidative phosphorylation (Fig. 2.10, Fig. 2.11). However, at higher concentration, 2,3-dimercaptopropanol (1 μmol/mg protein) was found to reverse inhibition by DBCT (Fig. 2.12), triphenyl tin chloride tributyl tin chloride, and dibutyl tin dichloride (Table 2.7) 80-100%. In all cases of organotin inhibition (except inhibition by dibutyl tin dichloride) of OS-ATPase, activity was found to be irreversible by dithiothreitol or dihydrolipoic acid (Table 2.7).

The inhibition of the ATP-driven NADH/NADP transhydrogenase activity of beef heart submitochondrial particles by most of the organotin compounds studied, was found to be reversible by dithiols. In the cases of DBCT (Fig. 2.13), dibutyl tin dichloride (Fig. 2.14) and triphenyl tin (Fig. 2.15) inhibition of the ATP-driven transhydrogenase activity, complete reversal was obtained with 300 nmol/mg, 100 nmol/mg and 500 nmol/mg protein of 2,3-dimercaptopropanol respectively. In most cases dithiothreitol and dihydrolipoic acid were found to be ineffective as reversal agents at 500 nmol/mg protein (Table 2.8).

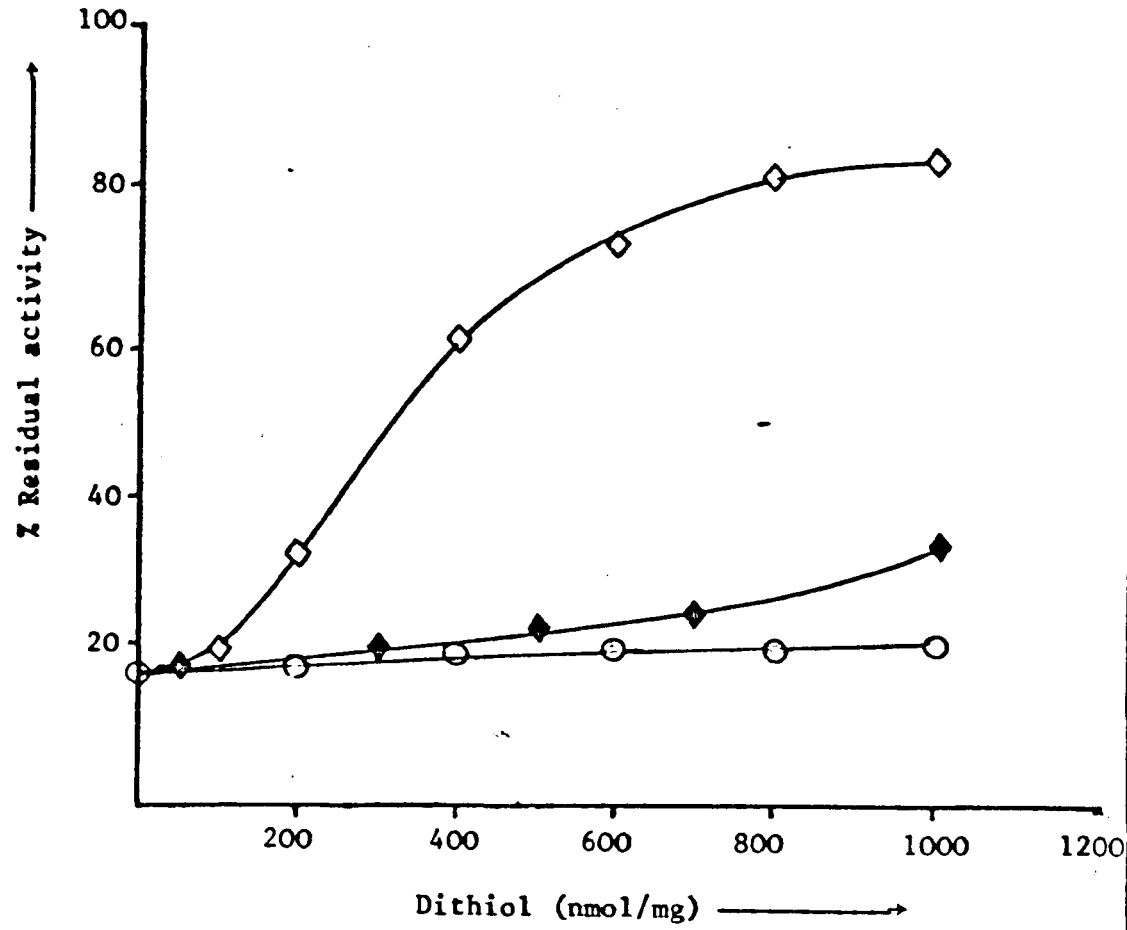


Fig. 2.12 Dithiol reversal of DBCT inhibited OS-ATPase activity in beef heart submitochondrial particles

Submitochondrial particles suspend at 10 mg protein/ml, in 0.25 M sucrose, 10 mM Tris-Cl, 1 mM EDTA buffer wwere preincubated with DBCT (4 nmol/mg) at 4° C for 30 minutes. The DBCT treated particles were then incubated at various concentrations of dithiols at 4° C for 30 minutes. The ATPase activity was assayed as described in Methods. The specific ATPase activity of the starting submitochondrial particles was 2.7 µmol ATP hydrolysed/ min/mg. Experimental data were expressed in the figure as a percent of this original value. (O), dithiothreitol; (◆), dihydrolipoic acid; (◇), 2,3-dimercaptopropanol. Each point is the average of 4 duplicates.

2.7 Dithiol reversal of DBCT and DBT inhibition of OS-ATPase activity in mitochondria and submitochondrial particles

Additions	% OS-ATPase activity in:	
	Mitochondria	SMP
DBCT (5 nmol/mg)	0.0	18.0
DBCT + 2,3-dimercaptopropanol (1 µmol/mg)	100.0	80.0
DBCT + dihydrolipoic acid (1 µmol/mg)	0.0	32.0
DBCT + dithiothreitol (1 µmol/mg)	0.0	18.0
DBT (6.6 nmol/mg)	0.0	
DBT + 2, 3-dimercaptopropanol (1 µmol/mg)	100.0	100.0
DB + dihydrolipoic acid (1 µmol/mg)	100.0	100.0
DBT + dithiothreitol (1 µmol/mg)	10.0	20.0
Ve_{2283} (2 nmol/mg) + 2,3-dimercaptopropanol (1 µmol/mg)	0.0	0.0
Oligomycin (5 nmol/mg) + 2,3-dimercapto-propanol (1 µmol/mg)	0.0	0.0

Mitochondria and submitochondrial particles suspended at 10 mg/ml were preincubated with DBCT (5 nmol/mg); DBT, (6.6 nmol/mg); Ve_{2283} (2 nmol/mg) or oligomycin 5.0 nmol/mg as described in Table 2.7. The particles were then incubated with dithiols at 1 µmol/mg for 30 minutes at 4° C, and the ATPase activity assayed as in methods. The specific ATPase activities of controls were: in mitochondria 0.85 µmol ATP hydrolysed/min/mg; in SMP 2.6 µmol ATP hydrolysed/min/mg. Experimental data have been expressed in the Table as a per cent of these original values. Each value is the mean of 4 duplicates.

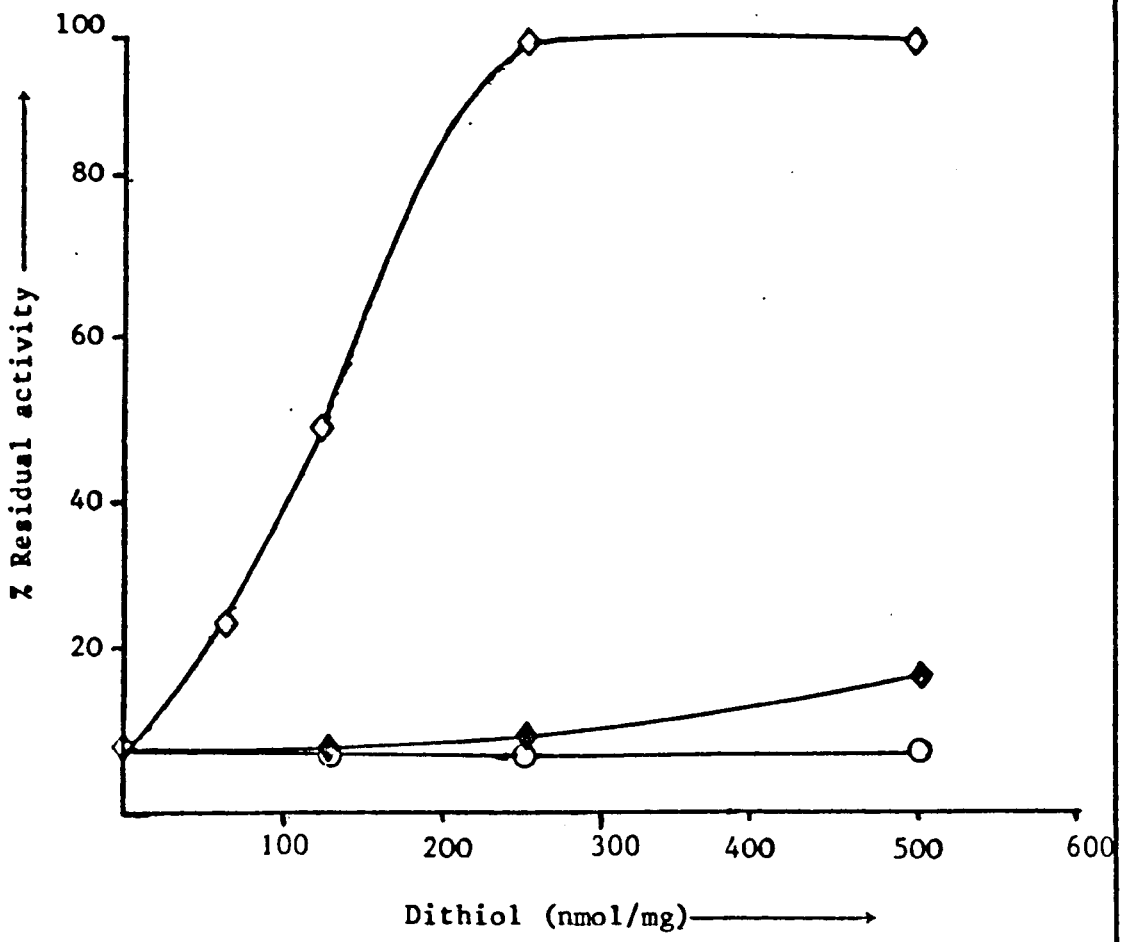


Fig. 2.13 Dithiol reversal of DBCT inhibition of ATP-driven transhydrogenase activity in submitochondrial particles

Submitochondrial particles were preincubated with DBCT (10 nmol/mg) treated with dithiols and ATP-driven transhydrogenase activity assayed as described in Fig. 2.14. Specific activity of starting SMP's was 38.0 nmol NADPH formed/min/mg. Experimental data have been presented in the figure as a per cent of this original value. (◇), 2,3-dimercaptopropanol; (◆), dihydrolipoic acid; (O), dithiothreitol. Data points are average of duplicates.

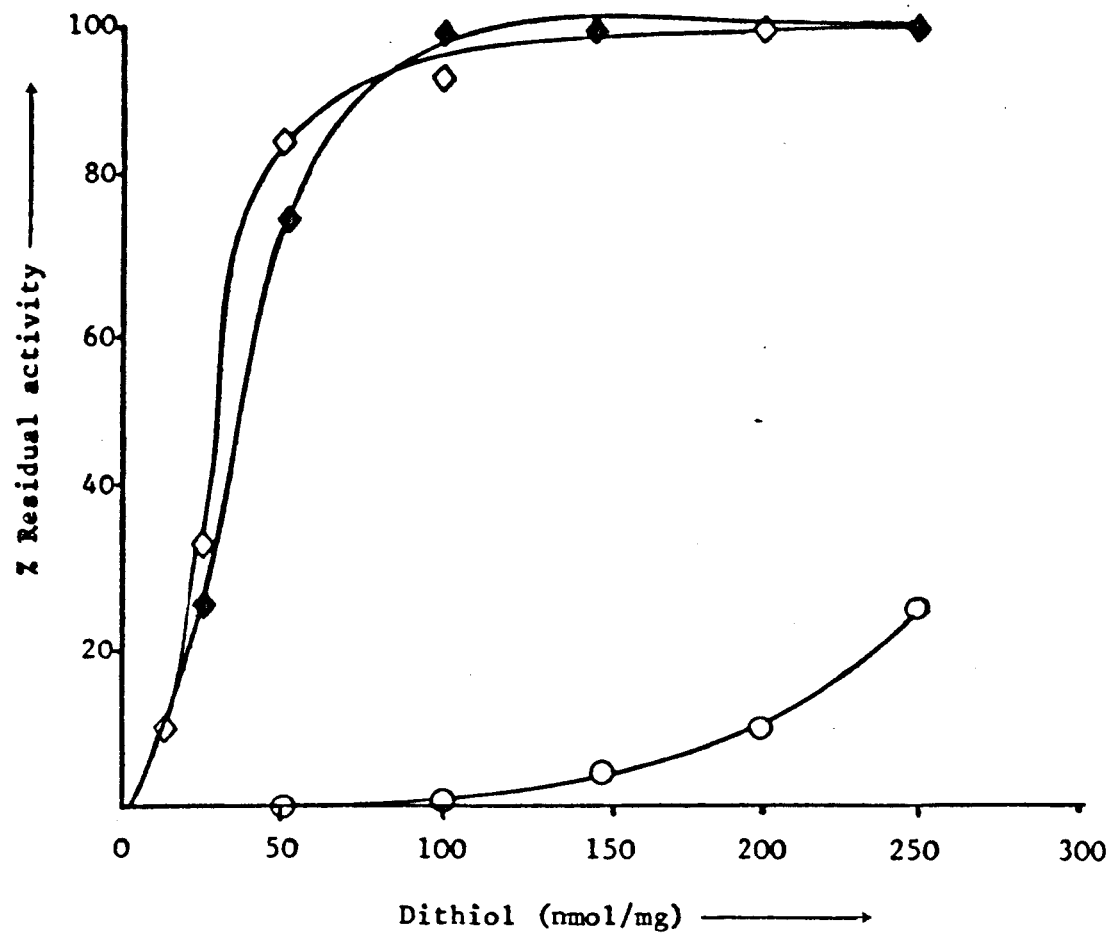


Fig. 2.14 Dithiols reversal of dibutyl tin dichloride (DBT) inhibited ATP-driven transhydrogenase activity in beef heart submitochondrial particles

Submitochondrial particles suspended at 10 mg protein/ml was pre-incubated with DBT (10 nmol DBT/mg) and then treated with dithiols as described in Fig. 2.11. The ATP-driven transhydrogenase activity was assayed as in Methods. Specific transhydrogenase activity of starting SMP's was 38.0 nmol NADPH formed/min/mg. Experimental data have been presented in the figure as a per cent of this original value. Each data point is the average of duplicate. (◆), 2,3-dimercaptopropanol; (◇), dihydrolipoic acid; (○),dithiothreitol.

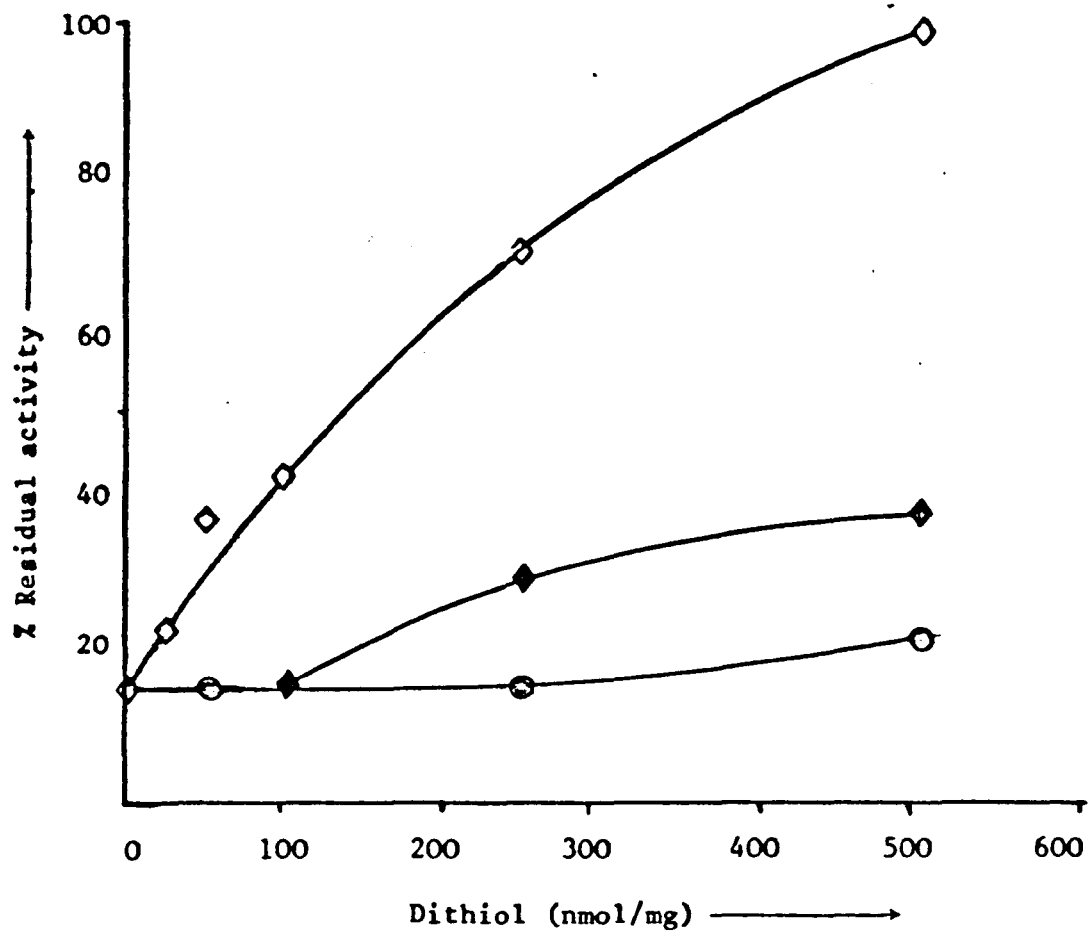


Fig. 2.15 Dithiols reversal of triphenyl tin (TPT) inhibition of ATP-driven transhydrogenase in beef heart submitochondrial particles

Submitochondrial particles were preincubated with TPT (8.0 nmol/mg) then treated with dithiols as described in Fig. 2.11. Transhydrogenase activity assayed as in Methods. Specific transhydrogenase activity of starting SMP's was 38.0 nmol NADPH formed/min/mg. Experimental data have been presented in the figure as a per cent of this original value. (◇), 2,3-dimercaptopropanol; (◆), dihydrolipoic acid; (○), dithiothreitol. Data points are average of duplicates.

Table 2.8 Dithiol reversal of DBCT and DBT inhibition of ATP-driven transhydrogenase in submitochondrial particles (SMP's)

Additions	% ATP-driven Transhydrogenase activity
None	100.0
DBCT (10 nmol/mg)	0.0
DBCT + 2,3-dimercaptopropanol (500 nmol/mg)	100.0
DBCT + dihydrolipoic acid (500 nmol/mg)	18.0
DBCT + dithiothreitol (500 nmol/mg)	10.0
DBT (10 nmol/mg)	0.0
DBT + 2,3-dimercaptopropanol (500 nmol/mg)	100.0
DBT + dihydrolipoic acid (500 nmol/mg)	100.0
DBT + dithiothreitol (500 nmol/mg)	20.0
Ve_{2283} + 2,3-dimercaptopropanol (500 nmol/mg)	0.0
Oligomycin + 2,3-dimercaptopropanol (500 nmol/mg)	0.0

Submitochondrial particles suspended at 10 mg/ml in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA, were preincubated with DBCT; DBT, Ve_{2283} and oligomycin 10, 10, 2 and 5 nmol/mg respectively for 30 minutes at 4° C. The particles were then incubated with dithiols at 500 nmol/mg at 4° C for 30 minutes and the ATP driven transhydrogenase activity assayed as described in methods.
The specific ATP-driven transhydrogenase activity of the original SMP's was 42.5 nmol NADPH formed/min/mg. Experimental data have been expressed as a per cent of this original value. Each value presented is the mean of 3 duplicates.

Table 2.9 Sensitivity of dithiol reversed DBCT, and DBT inhibition of succinate driven ATP synthesis and ATP-dependent reactions in SMP's to inhibitors and uncouplers of oxidative phosphorylation

Additions	% ATP Synthesis	% OS-ATPase activity	% ATP-driven Transhydrogenase
None	100.0	100.0	100.0
DBCT (15 nmol/mg)	15.0	0.0	0.0
DBCT + BAL (400 nmol/mg)	100.0	60.0	100.0
DBCT + BAL + TTFB (2 µg/mg)	0.0	65.0	0.0
DBCT + BAL + Ve_{2283} (2 nmol/mg)	0.0	0.0	0.0
DBCT + BAL + oligomycin (5 nmol/mg)	0.0	0.0	0.0
DBT (30 nmol/mg)	18.0	0.0	0.0
DBT + BAL (400 nmol/mg)	100.0	100.0	100.0
DBT + BAL + TTFB (2 µg/mg)	0.0	100.0	0.0
DBT + BAL + Ve_{2283} (2 nmol/mg)	0.0	0.0	0.0
DBT + BAL + oligomycin (5 nmol/mg)	0.0	0.0	0.0

Submitochondrial particles (SMP's) suspended at 10 mg/ml were preincubated with DBCT (15 nmol/mg) or DBT (30 nmol/mg) under the conditions described in Table 2.7. The particles were then incubated with 2,3-dimercaptopropanol (BAL) at 400 nmol/mg for 30 minutes at 4° C. Aliquots were then removed and assayed for OS-ATPase, ATP-driven transhydrogenase and succinate drivenoxidative phosphorylation as described in Methods. The specific activities of the starting SMP's were: OS-ATPase, 2.4 µmol ATP hydrolysed/min/mg; transhydrogenase 42.5 nmol NADPH formed/min/mg; succinate driven oxidative phosphorylation, 80 nmol ATP synthesised/min/mg. Experimental data have been expressed as a per cent of these original values. Each value presented is the average of duplicates. TTFB, Ve_{2283} and oligomycin were added as methanolic solutions in the amount indicated directly to the assay system.

However, dihydrolipoic acid was found to be an effective reversal agent of dibutyl tin dichloride inhibition of the transhydrogenase activity. With dibutyl tin dichloride inhibition, dihydrolipoic acid was found to be as effective a reversal agent as 2,3-dimercapto-propanol (Fig. 2.14); dihydrolipoic acid at 100 nmol/mg protein, giving 90-100% reversal of dibutyl tin dichloride inhibited ATP-driven transhydrogenase. As in the cases of oxidative phosphorylation and OS-ATPase, inhibition of ATP driven transhydrogenase by Ve_{2283} was irreversible by dithiols.

The oxidised form of lipoic acid, and monothiols such as glutathione, cysteine and 2-mercaptoethanol were found to be totally ineffective in reversing trialkyl tin inhibition of OS-ATPase oxidative phosphorylation or ATP-driven transhydrogenase activity.

The results presented in Table 2.9, show that the dithiol reversed trialkyl tin inhibition of oxidative phosphorylation and ATP-driven transhydrogenase were inhibited by oligomycin, Ve_{2283} and the uncoupler TTFB, but not by the addition of more ($\equiv$ 10 nmol/mg) DBCT or DBT when the dithiol was present in excess. The reversed OS-ATPase activity was similarly inhibited by oligomycin and Ve_{2283}. Prior treatment of submitochondrial particles with 2,3-dimercapto-propanol (500 nmol/mg) was found to provide protection against inhibition of oxidative phosphorylation, ATP-driven transhydrogenase and OS-ATPase activity by DBCT and DBT, but not inhibition by oligomycin or Ve_{2283}.

2.6 DISCUSSION

The results presented in this Chapter have shown that DBCT, Ve_{2283} and most of the trialkyl tin compounds studied, were

potent inhibitors of mitochondrial energy-transfer reactions. However, unlike oligomycin and DCCD inhibition of the energy-transfer reactions, inhibition by most trialkyl tins examined was reversible by dithiol (Tables 2.6-2.9). The mechanism(s) by which the trialkyl tins inhibits these processes is (are) not known, but the experimental findings seem to suggest that the modes of inhibition of respiratory dependent and the ATP-dependent reactions were different. The results obtained indicate, that while an oligomycin-like activity is responsible for the inhibition of OS-ATPase and ATP-driven transhydrogenase, an uncoupling mode of action is responsible for the inhibition of oxidative phosphorylation and succinate-driven transhydrogenation (see Fig. 2.1).

Aldridge and Street (140, 162, 163) have proposed that inhibition of the OS-ATPase was brought about by penta-coordinate binding of the trialkyl tin compounds at the inhibitor binding sites. The penta-coordinate binding proposal, provides explanations for the relative ineffectiveness of tetrabutyl tin and the potency of dibutyl tin dichloride (DBT) as inhibitors of the OS-ATPase. The presence of the fourth butyl group in tetrabutyl tin, sterically hinders penta-coordination and therefore its binding to the inhibitor binding site. However, dibutyl tin dichloride can undergo penta-coordination (with a hydroxyl group forming the 5th ligand) and will therefore bind the inhibitor binding sites. The ability of a trialkyl tin compound to undergo penta-coordination is not the only criterion which determines its inhibitory potency; the nature of the alkyl groups is a very important factor. Aldridge and Street have proposed, that for efficient interaction of trialkyl tins with the binding site and therefore efficient inhibition of the OS-ATPase, a certain minimum size for the alkyl group is required (143). Thus, according to the 'penta-coordinate hypothesis', the potency of the trialkyl

tin compound as an inhibitor of the OS-ATPase would depend on how closely the molecule approaches a certain optimum shape and size. For example, although trimethyl tin chloride undergoes penta-coordination, it is a poor inhibitor of the OS-ATPase of beef heart (Table 2.1), rat liver (139) and yeast mitochondria (142). Aldridge et al. (143) have proposed that this is due to the low binding affinity of the trimethyl tin (which is 10 times lower than that of triethyl tin in rat liver mitochondria) for the inhibitor binding site, and is a result of the size of the methyl group. Penta-coordination is supported by the fact that the penta-coordinate compound Ve_{2283} is a good inhibitor of the OS-ATPase.

Aldridge et al. (143) have postulated that the mechanism of trialkyl tin binding to the inhibitor site on the OS-ATPase, involves the formation of tin-nitrogen (Sn-N) linkages between the trialkyl tin and paired histidine residues (Fig. 2.16); similar to that proposed by Rose (164) for the binding of triethyl tin to rat haemoglobin; and by Davidoff and Carr (165) for the binding of triethyl tin to pyruvate kinase. However, the lack of involvement of tin-nitrogen linkage in the mechanism of trialkyl tin inhibition, has been demonstrated by Gould (166); who has shown that triphenyl tin inhibition of OS-ATPase functions cannot be reversed by large excess of exogenous histidine. Furthermore, triphenyl germanium, which unlike triphenyl tin does not under penta-coordination (i.e. form 5-coordination compounds) in imidazole model systems, exhibits inhibitory properties identical to those seen with triphenyl tin (166). This is inconsistant with the proposals of Aldridge et al. (143), Rose (164) and Davidoff and Carr (165). However, it is known that trialkyl tin compounds form polymeric coordination complexes with certain heterocyclic nitrogen-coordinated ring

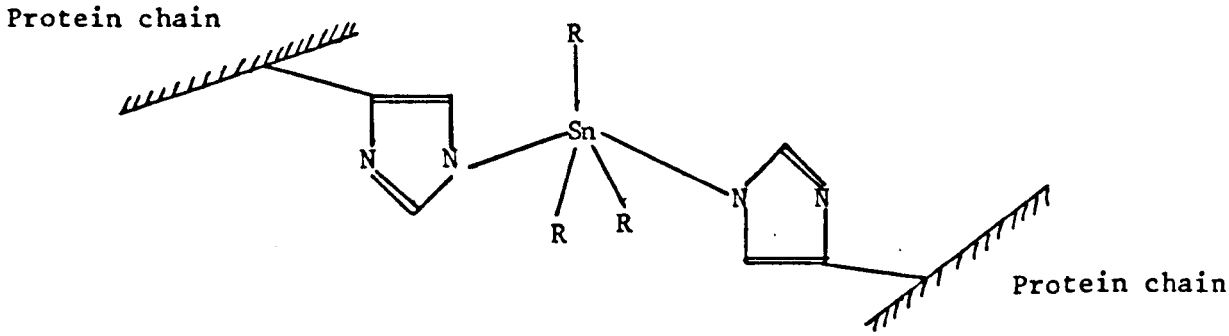


Fig. 2.16 Proposed structure for trialkyl tin binding in haemoglobin, pyruvate kinase, OS-ATPase (143, 164, 165).

The trialkyl tin is proposed to form a pentacoordinate complex with the histidines at the binding site.

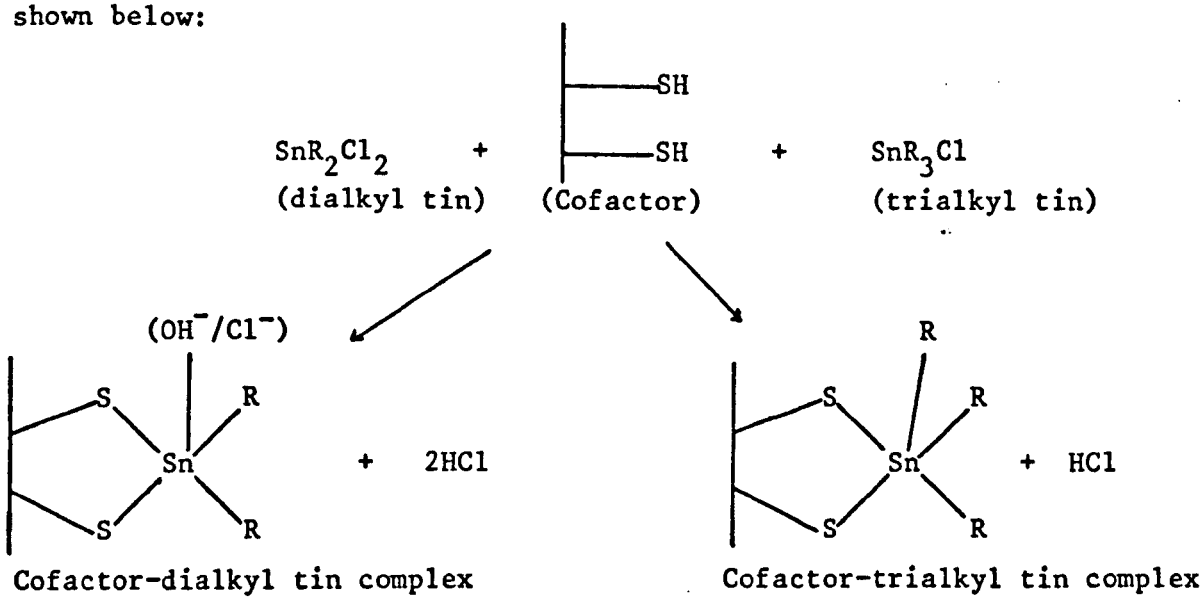
complexes such as imidazole (167, 168).

The results presented above (Table 2.6-2.9, Fig. 2.10-2.15) have shown that the inhibition of oxidative phosphorylation, OS-ATPase and ATP-driven transhydrogenase activities by most organotin compounds can be effectively reversed by dithiols, but not by monothiols. Similar findings have been reported by Cain et al. (159) who have shown that inhibition by dibutyl tin dichloride could be reversed by dithiols. Gould (169) has also shown that triphenyl tin inhibition of photophosphorylation and the increased proton permeability in CF_1 deficient chloroplast were specifically reversed by dithiols. Although these results do not provide conclusive proof, they can nevertheless be taken as suggestive evidence for a role of vicinal dithiol residues in the mechanism of organotin inhibition of OS-ATPase function.

Reversal of organotin inhibition of mitochondrial functions by dithiols have been proposed to be due to:

1. a 'scavenging' effect, resulting in the removal of the organotin from the system;
2. and/or the displacement of the cofactor to which the organotin binds by dithiol interchange reaction;
3. and/or the replacement of the cofactor by the added dithiol (150, 159).

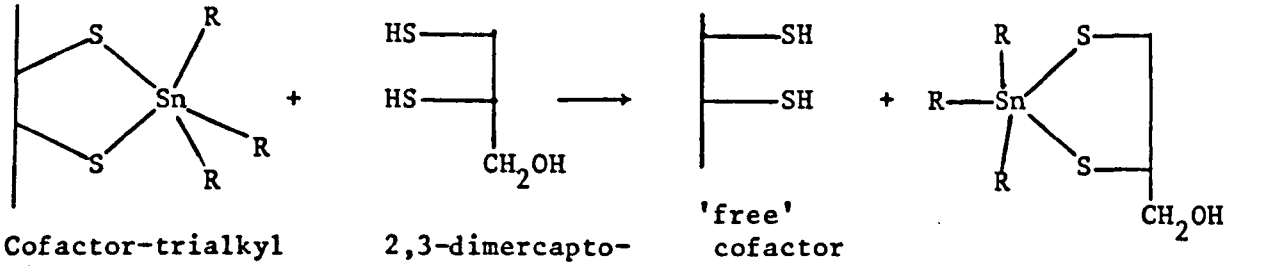
The proposals were put forward by Aldridge et al. (161) and Griffiths et al. (150, 159) who proposed that organotins inhibit mitochondrial functions by binding to a cofactor containing a vicinal dithiol, such as dihydrolipoic acid, which acts as a high energy chemical intermediate. Trialkyl tins and dialkyl tins are proposed to act in a similar manner to arsenite and Lewisite; forming very stable 5 or 6-membered ring, penta-coordinate complexes with the cofactor, as shown below:



Cofactor-dialkyl tin complex

Cofactor-trialkyl tin complex

Thus in analogy with the reversal of arsenite or Lewisite inhibition by dithiol interchange (170), reversal of dialkyl tin and trialkyl tin by dithiols can be represented as follows:



Cofactor-trialkyl tin complex + 2,3-dimercapto-propanol → 'free' cofactor + trialkyl tin dimercaptopropanol complex

The specific reversal of DBCT inhibition of mitochondrial oxidative phosphorylation, OS-ATPase and ATP-driven transhydrogenase activities by dihydrolipoic acid reported by Cain et al. (159) led Grififths to suggest that dihydrolipoic acid might be the dithiol cofactor proposed in the equations above (150). However, contrary to the finding of Cain et al. (159), 2,3-dimercaptopropanol and not dihydrolipoic acid, was found to be the best reversal agent in all cases of organotin inhibition. In most cases 2,3-dimercaptopropanol was found to be 15-25 times more effective than dihydrolipoic acid as a reversal agent (Figs. 2.10-2.15; Tables 2.6-2.9). Only in the reversal of dibutyl tin dichloride inhibition of ATP-driven transhydrogenase, OS-ATPase and oxidative phosphorylation (Fig. 2.14, Tables 2.6-2.8) were they found to be equally effective. Dithiothreitol and monothiols were found to be ineffective as reversal agents in most cases. The fact that 2,3-dimercaptopropanol is a better reversal agent than dihydrolipoic acid and dithiothreitol, might be due to its electrically neutral and hydrophobic nature; and/or, that the rate of oxidation of its thiol groups is much slower. Results presented in Chapter 3 show that most of the organotin is present in the lipid fraction of the mitochondrial membrane. The hydrophobic nature of 2,3-dimercaptopropanol thus allows easy access to the organotin. The hydrophilic nature of dihydrolipoic acid combinedwith the fact that it has a negatively

charged carboxylic group (under the conditions of the experiment), hinder the availability of the dihydrolipoic acid to the organotin in the membrane.

Although the results on dithiol reversal of organotin inhibition of energy coupling reactions are consistant with the proposal that a vicinal dithiol is involved in the binding of trialkyl tins to the inhibitor site, they do not support the proposal of Griffiths (159) that dihydrolipoic acid is the vicinal dithiol.

Although DBCT, Ve_{2283} and the other trialkyl tins studied were found to be potent inhibitors of the OS-ATPase and succinate driven oxidative phosphorylation, the two processes exhibit different degrees of sensitivity to these organotins. In all cases, the OS-ATPase was found to be 6-10 times more sensitive to organotin inhibition than oxidative phosphorylation (Tables 2.1-2.3) and Figs. 2.2-2.7). The results presented on Fig. 2.6 and Fig. 2.7 show that it is possible to inhibit the OS-ATPase activity by 90% in mitochondria and submitochondrial particles with DBCT, without significantly affecting succinate driven oxidative phosphorylation. These results indicate that the modes of trialkyl tin inhibition of oxidative phosphorylation and OS-ATPase were different, and/or that ATP synthesis and ATP hydrolysis occurs at different sites on the OS-ATPase complex. Separate sites for ATP synthesis and ATP hydrolysis have been suggested by Penefsky (171) and Pedersen et al. (81, 172) to exist on the OS-ATPase, based on the observation that AMP(PNP) inhibited ATP hydrolysis but did not affect oxidative phosphorylation. In the 'separate site model' it is assumed that the inhibitor, DBCT, or AMP(PNP) binds preferentially to the ATP hydrolysing site, thus inhibiting the OS-ATPase activity (81, 172). However, a 'conformational model' could also be used to explain the differential sensitivity of the ATP hydrolytic and the

ATP synthetic reactions to trialkyl tins. In this 'model' ATP synthesis and hydrolysis occurs at the same catalytic site on the enzyme; however, whether ATP synthesis or hydrolysis occurs depends upon the conformation of the enzyme. The binding of DBCT or another trialkyl tin to the inhibitor site, induces the attainment of that conformation that favour synthesis of ATP and inhibition of ATP hydrolysis. In both the 'conformational' and 'separate site' models, inhibition of ATP synthesis at the higher concentrations (15-20 nmol/mg protein) of trialkyl tins, would be due to uncoupling of oxidative phosphorylation. Uncoupling being brought about either through the detergent effect of the trialkyl tins on the mitochondrial membrane, or by discharging of the proton gradient through their ability to catalyse Cl^-/OH^- exchange across the membrane (140). This is supported by the finding (Fig. 2.8) that at 15-20 nmol trialkyl tin/mg protein succinate driven transhydrogenation is also inhibited.

The proposals of Griffiths (150, 150) of a pool of cofactor involved in energy coupling which are titratable by trialkyl tins, cannot be used to explain the differential inhibition of the OS-ATPase and oxidative phosphorylation by trialkyl tins, or the significant increase (10-20%) in the rate of ATP synthesis in submitochondrial particles at 0.5-0.8 nmol DBCT/mg protein (Fig. 2.7), since the same catalytic site is involved in both reactions. Such an increase has also been obtained by G. Solaini (173) using tributyl tin as the inhibitor. The increase could be due to either the inhibition of the ATPase which would otherwise hydrolyse the ATP synthesised during oxidative phosphorylation (an apparent increase), or the inhibition of proton leakage resulting in increase coupling efficiency (a real increase).

The differential sensitivity of the OS-ATPase and oxidative phosphorylation to trialkyl tin inhibition, if it is not artificial, is consistant with the proposals that trialkyl tin inhibition of ATP synthesis and ATP hydrolysis are brought about by two different mechanisms and that the OS-ATPase is at least bifunctional. The findings, that, in general, 20-30 times more 2,3-dimercaptopropanol was needed to reverse trialkyl tin inhibition of the OS-ATPase activity than was needed to reverse trialkyl tin inhibition of oxidative phosphorylation sensitive to oligomycin and uncoupler (Table 2.9), corroborate the differential effect of trialkyl tins on the two processes and provide support for different mechanisms of inhibition of the two processes.

The most 'interesting' of the organotin compounds examined is Ve_{2283}. This compound is the most potent organotin so far examined. Its inhibition of mitochondrial energy-transfer reactions is irreversible by dithiols or monothiols. Using the 2,6-dichlorophenol-indophenol technique described by Aldridge and Cremer (175), Carver (174) has shown that Ve_{2283} does not react with dithiols or monothiols. This compound (especially radioactively labelled) because of its specificity, will be very useful in mapping the inhibitory binding site(s) of organotins, and help to elucidate their mode of inhibition.

2.7 CONCLUSION

The results presented in this Chapter have shown that DBCT, DBT, Ve_{2283} and most of the trialkyl tin compound studied, were potent inhibitors of mitochondrial energy-transfer reactions. However, unlike oligomycin and DCCD inhibition of the energy-transfer reactions,

inhibition by most organotins examined was reversible by dithiols (Tables 2.6-2.9). The mechanism(s) by which the organotins inhibit these processes is (are) not known, but the experimental findings seem to suggest that the modes of inhibition of oxidative phosphorylation and OS-ATPase activity were different. The results obtained, indicate that while an oligomycin-like activity is responsible for the inhibition of OS-ATPase and ATP-driven transhydrogenase, an uncoupling mode of action is responsible for the inhibition of oxidative phosphorylation. The results also suggest that ATP synthesis and hydrolysis occur at two different sites.

CHAPTER 3

CHARACTERIZATION OF DIBUTYLCHLOROMETHYL TIN CHLORIDE BINDING SITES IN BEEF HEART MITOCHONDRIA

3.1 INTRODUCTION

Inhibitors of oxidative phosphorylation and OS-ATPase activities in mitochondria, such as dicyclohexylcarbodiimide (DCCD), oligomycin and trialkyl tins can provide insight into the molecular requirements for these processes, provided sufficient information about their mechanisms of inhibition are known. For example, it has been shown that DCCD and oligomycin initiates inhibition of oxidative phosphorylation and OS-ATPase activities by binding covalently (177) and non-covalently to their respective, specific binding proteins. However, although trialkyl tins exhibit similar inhibitory properties, they have never been found to bind specifically to any protein component of the OS-ATPase complex.

Previous studies on the binding of trialkyl tin compound to rat liver mitochondrial membrane by Aldridge and Street (143), revealed the presence of high and low affinity binding sites for trialkyl tins on mitochondrial membrane. The oligomycin-like effect of trialkyl tin was correlated with the high affinity binding site, which for triethyl tin was of the order of 0.8 nmol/mg protein. However, the high affinity binding site has never been shown to be present on any specific component of the oxidative phosphorylation system. Recent experiments by Griffiths et al. (149) using [^{3}H]-DBCT, (DBCT is proposed to behave as a specific covalent inhibitor of mitochondrial ATPase and oxidative phosphorylation) have suggested that trialkyl tins bind to dithiol components, present on the OS-ATPase complex. The [^{3}H]-DBCT labelled complex has been

isolated, and was found to be a small non-protein, hydrophobic molecule, distinct from the DCCD and oligomycin binding proteolipids.

In this Chapter, the binding of [^{3}H]-DBCT to submitochondrial particles is examined and the binding parameters compared with those derived from inhibition studies on OS-ATPase and oxidative phosphorylation. The'[^{3}H]-DBCT-X' component was isolated, its R_f value in various solvent systems estimated, and an attempt made to identify it using mass spectrometry.

3.2 MATERIALS

[^{3}H]-DBCT was a gift from Professor B. Beechey, Shell Research Ltd., Sittingbourne, Kent.
Trialkyl tins, lipoic acid, dihydrolipoic acid, were obtained as previously stated in Materials section of Chapter 2. Precoated silica gel plates for analytical (TLC - silica gel $60F_{254}$ 20 cm x 20 cm x 0.2 mm) and preparative (PSC - silica gel, 20 cm x 20 cm x 2 mm) chromatography were purchased from E. Merck, Darmstadt. All organic solvents were of AnalaR grade and were redistilled before use.

3.3 METHODS

Beef heart submitochondrial particles were prepared as described in the Methods section of Chapter 2.

[^{3}H]-DBCT binding study: beef heart submitochondrial particles suspended in 0.25 M sucrose, 10 mM tris-Cl, 1 mM EDTA, pH 7.5 (at 2 mg protein/ml) were incubated with various [^{3}H]-DBCT concentrations for 16 hours at 4° C. [^{3}H]-DBCT was added as a

methanolic solution. The suspensions were centrifuged at 100,000 g in the 40.3 rotor of the Beckman ultracentrifuge for 30 minutes at 5° C to 'pellet' the submitochondrial particles. Samples of the supernatant were taken for scintillation counting and the remaining supernatant discarded. The 'pellet' was solubilised by leaving overnight in 1.0 ml of 2% (w/v) Triton X-100. The radioactivity was determined in 4 ml of a Triton X-100 based scintillant cocktail (1 litre toluene, 0.5 litre Triton X-100, 7 g butyl PBD). Standards were counted with each batch of samples. Under these conditions 70-75% efficiency of counting was achieved. There was no significant binding of [^{3}H]-DBCT to the cellulose nitrate tubes. The distribution of the [^{3}H]-DBCT obtained on the basis of the equilibrium binding studies was analysed according to Scatchard (176).

Competitive binding experiments were carried out using 'cold' DBCT, tributyl tin chloride, Ve_{2283} and dibutyl tin dichloride. In these experiments submitochondrial particles were preincubated with 'cold' DBCT, tributyl tin, Ve_{2283} or dibutyl tin dichloride (at 15 nmol/mg protein) for 3 hours, before addition of various concentrations of [^{3}H]-DBCT. The suspensions were centrifuged at 100,000 g in the 40.3 rotor of the Beckman ultracentrifuge for 30 minutes at 5° C to pellet the submitochondrial particles. The pellet was solubilised in 1.0 ml 2% (w/v) Triton X-100, and the radioactivity determined as described above.

Extraction of [^{3}H]-DBCT-X from submitochondrial particles with acetone [Svoboda et al. (5)]

Submitochondrial particles (2 mg/ml) were treated with varying concentrations of [^{3}H]-DBCT as described previously. To 0.5 ml of the suspension 0.5 ml 10% TCA was added. This mixture was filtered through a single Whatman GF/C filter, and the residue

left on the filter washed at a very low flow-rate (30-40 ml/min) with 45 ml of the following solutions:

(a) 0.25 M sucrose, 10 mM tris-Cl, 1 mM EDTA, pH 7.4, and

(b) 10% water in acetone.

As much as 90-95% of the protein remained on the filters. The filters were dried at 30° C on a hot plate for 15 minutes and the radioactivity determined by dropping the filter in a scintillation vial containing 4.0 ml of the liquid scintillation cocktail mentioned above.

Isolation of the DBCT-X complex

Extraction of the DBCT-X complex from submitochondrial particles with chloroform/methanol (2:1, v/v) was carried out essentially as described by Cattell et al. (181). Submitochondrial particles (600.0 mg) suspended at 20 mg protein/ml was incubated with [^{3}H]-DBCT at 20 nmol/mg protein for 16 hours. The submitochondrial particles were pelletted by centrifugation at 100,000 g in the 30 rotor of the Beckman ultracentrifuge for 90 minutes. The pellet was resuspended in an equal volume of 0.25 M sucrose, 10 mM tris-Cl, 1 mM EDTA pH 7.4 buffer and recentrifuged as before. This process was repeated three times, after which the [^{3}H]-DBCT treated submitochondrial particles were resuspended in an equal volume of the same buffer, the suspension added to 25 volume of chloroform/methanol (2:1, v/v) and stirred at room temperature overnight. The mixture was filtered through a Whatman GF/C filter (under pressure if necessary) and the filtrate washed with 1/5 vol. water. The emulsion is allowed to separate into two distinct layers, and the lower chloroform/methanol layer isolated. The washed chloroform/methanol extract was vacuumed down to 1/5 vol. using a rotory evaporator, 5 vol. of ice cold diethyl ether added and the mixture left overnight at -20° C. The precipitated proteolipids were removed by centrifugation and

redissolved in chloroform. The supernatant from the centrifugation step was vacuumed down to minimal volume and the residue redissolved in chloroform. This is DBCT-X. Samples of this solution were subjected to analytical and preparative t.l.c. in various solvent systems on silica gel t.l.c. plates. The [^{3}H]-DBCT-X was purified by preparative thin layer chromatography on silica gel in $CHCl_3$/MeOH (2:1) solvent system.

Mass spectral analysis of the purified DBCT-X component was carried out by Mr. I. Katyal, Chemistry and Molecular Sciences Department, University of Warwick.

3.4 RESULTS

Fig. 3.1 shows the binding of [^{3}H]-DBCT to beef heart submitochondrial particles, and the effect of increasing concentration of the inhibitor on the OS-ATPase activity in these particles. The binding curve (▼——▼) indicates that the saturation of the DBCT binding sites were not obtained within the [^{3}H]-DBCT concentration range used. However, the percentage bound DBCT decreases from 80-95% of the DBCT added at 0.3-2.0 nmol/mg protein to 50-40% of the DBCT added at higher concentrations (4-30 nmol/mg protein). These results indicate that there are at least two different types of binding sites, a high affinity binding site which is apparently saturated at 2-3 nmol DBCT/mg protein and low-affinity binding site which is present at a much greater concentration.

Parallel measurements of the ATPase activity revealed that the half-maximum inhibition of the enzyme activity was reached at ∿0.9 nmol DBCT/mg protein. In these conditions ∿0.80 nmol of [^{3}H]-DBCT was bound per mg of submitochondrial particle protein (Fig. 3.1). Complete inhibition of the OS-ATPase activity was

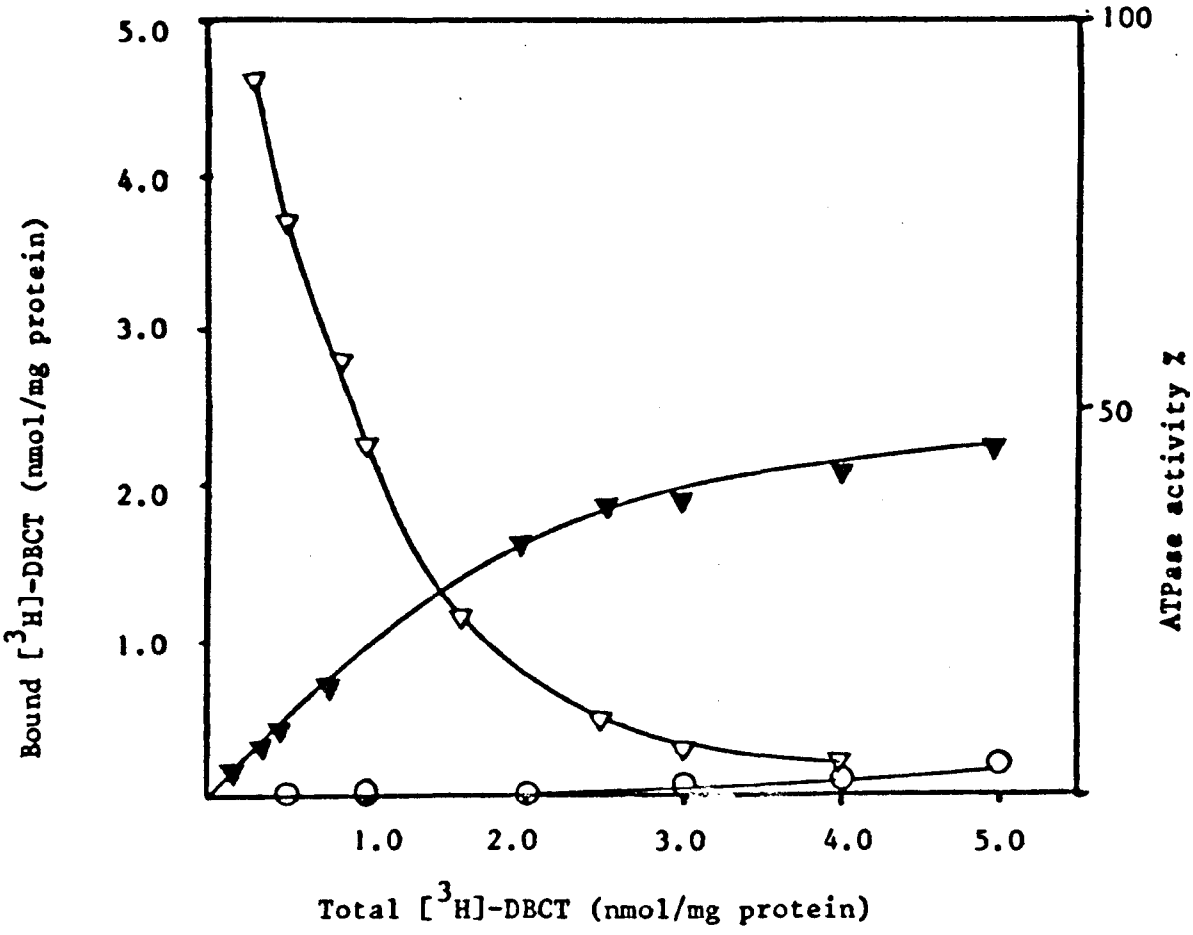


Fig. 3.1 [^{3}H]-DBCT binding to beef heart submitochondrial particles as a function of increasing concentrations of [^{3}H]-DBCT

Submitochondrial particles suspended at 2 mg protein/ml in 0.25 M sucrose; 10 mM tris-Cl, pH 7.5; 1 mM EDTA, were preincubated with various concentrations of [^{3}H]-DBCT for 16 hours. 100 µg protein aliquots (50 µl) were removed and assayed for ATPase activity. The suspensions were then analysed for bound [^{3}H]-DBCT as described in Methods (▼). Washing of [^{3}H]-DBCT treated particles with 10% water in acetone (O) was carried out by the method of Swoboda et al. (5) as described in Methods. The specific ATPase activity of the original submitochondrial particles is 2.5 µmol ATP hydrolysed/min/mg protein. The inhibition data (▽) are presented in the figure as a per cent activity of this original value. Each data point represents the average of 4 duplicates.

obtained at 2.5-3.0 nmol [^{3}H]-DBCT/mg protein, when 1.8-2.0 nmol of [^{3}H]-DBCT was bound per mg protein. Comparison of the OS-ATPase activity and [^{3}H]-DBCT binding curves, shows that DBCT is further bound even when maximum inhibition of the OS-ATPase activity is reached (Fig. 3.1).

When acetone-water was used for washing the submitochondrial particles, the shape of the binding curve was drastically modified (see Fig. 3.1). The bound [^{3}H]-DBCT was completely removed by the acetone-water treatment (Fig. 3.1). A solution of 10% water in acetone is known to extract the majority of the mitochondrial phospholipids (178). It could therefore be concluded that the [^{3}H]-DBCT is not bound to a protein component, but is bound, possibly to a phospholipid or a similar non-protein lipophilic component. The hydrophobic nature of the trialkyl tins in general, would indicate that such binding would occur non-specifically. The results also indicate that the inhibitory action of organotins is dependent upon a phospholipid, and is possibly manifested through a phospholipid-trialkyl tin complex.

The data presented in Table 3.1 shows the effect of tributyl tin, dibutyl tin dichloride Ve_{2283} and 'cold' DBCT on the binding of [^{3}H]-DBCT to submitochondrial particles. The results show that preincubation of submitochondrial particles with the above-mentioned trialkyl tins (at 15 nmol/mg protein) had no effect on the binding of [^{3}H]-DBCT to the particles. The pattern of binding in the presence of tributyl tin, Ve_{2283} and dibutyl tin dichloride was similar, but not identical to that in the absence of the added organotins; that is, below 2 nmol/mg 80-90% of the [^{3}H]-DBCT was bound whereas, at DBCT concentration greater than 3 nmol/mg protein, less than 60% remained bound (Fig. 3.2). When 'cold' DBCT was the added trialkyl tin, the pattern of binding was different, only 40-50% of the added [^{3}H]-DBCT

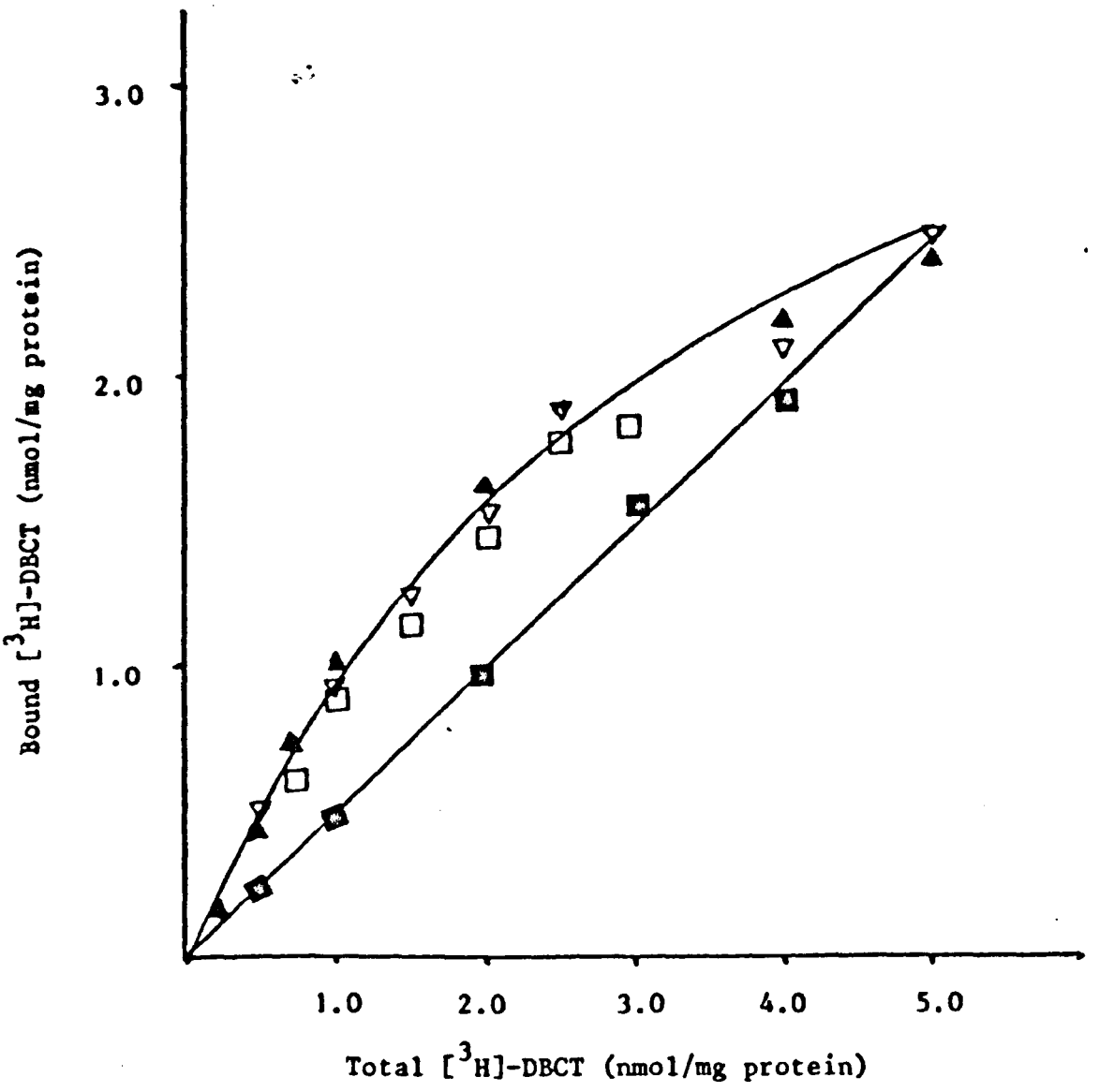


Fig. 3.2 The effect of various trialkyl tins on [^{3}H]-DBCT binding to submitochondrial particles

Submitochondrial particles suspended at 2.0 mg protein/ml were preincubated with dibutyl tin dichloride (☐), tributyl tin chloride (▽) and 'cold' DBCT (■) at 15 nmol/mg protein for 16 hours at 4° C. The treated particles were then incubated with varying concentrations of [^{3}H]-DBCT for 16 hours at 4° C. The suspensions were then analysed for bound [^{3}H]-DBCT as described in Methods. Experimental data is presented in the figure as the amount of [^{3}H]-DBCT bound plotted against the total concentration. Each data point is the average of duplicates. Control binding curve, (▲).

Table 3.1 Effect of trialkyl tin compounds on [^{3}H]-DBCT binding to beef heart submitochondrial particles

Organotin	[^{3}H]-DBCT (nmol/mg protein) A	B	% inhibition of binding
None	1.0	0.96	-
Tributyl tin chloride (2 nmol/mg)	1.0	0.92	4.2
Tributyl tin chloride (5 nmol/mg)	1.0	0.95	1.0
Tributyl tin chloride (10 nmol/mg)	1.0	0.88	8.3
Tributyl tin chloride (15 nmol/mg)	1.0	0.85	11.5
Tributyl tin chloride (25 nmol/mg)	1.0	0.86	10.4
Dibytyl tin dichloride (5 nmol/mg)	1.0	0.94	2.0
Dibutyl tin dichloride (10 nmol/mg)	1.0	0.94	2.0
Dibutyl tin dichloride (20 nmol/mg)	1.0	0.80	16.7
Dibutyl tin dichloride (30 nmol/mg)	1.0	0.84	12.5
Ve_{2283} (2 nmol/mg)	1.0	0.94	2.0
Ve_{2283} (5 nmol/mg)	1.0	0.94	2.0
Ve_{2283} (10 nmol/mg)	1.0	0.90	6.2
Ve_{2283} (15 nmol/mg)	1.0	0.91	5.0

A ≡ Total [^{3}H]-DBCT (1.0 nmol/mg protein). B ≡ bound [^{3}H]-DBCT. Submitochondrial particles suspended at 2.0 mg protein/ml in 0.25 M sucrose; 10 mM tris-Cl, pH 7.5; 1 mM EDTA buffer, were preincubated for 3 hours at 4° C with various concentrations of tributyl tin, dibutyl tin dichloride and Ve_{2283}. The particles were then incubated with [^{3}H]-DBCT at 1.0 nmol/mg protein for 16 hours. The suspensions were analysed for bound [^{3}H]-DBCT as described in the Methods section. Each value is the mean of duplicates.

was bound and the percentage bound was independent of the concentration of the added [^{3}H]-DBCT over the range used (Fig. 3.2). The results of competition experiments thus indicate that DBCT does not bind to the same site as tributyl tin, Ve_{2283} or dibutyl tin dichloride.

For evaluation of the maximum number of binding sites (B_{max}) and the determination of the apparent dissociation constant (k_D), the data of [^{3}H]-DBCT binding were analysed according to Scatchard (176). The Scatchard plot (Fig. 3.3) reveals that there are clearly two classes of binding sites with different binding affinities. Analysis of the plot shows that the maximum number of high affinity binding sites is ∿2.0 nmol/mg protein, having an apparent dissociation constant of 2.86×10^{-7} M. The low affinity site has a concentration of approximately 33.44 nmol/mg protein and a K_D of 30.4 μM. The high affinity site clearly correlates with I_{50} value of 0.8 nmol/mg protein for the ATPase inhibition. This is also in agreement with the conclusion of Aldridge and Street (143) that the high affinity site in rat liver mitochondria represented the binding site responsible for the oligomycin-like effects of triethyl tin.

Fig. 3.4 is a Dixon plot (1/v against [DBCT]), of the OS-ATPase inhibition data presented in Fig. 3.1 (179). The plot shows that there are more than one binding site for DBCT; at least two sites are present. Extrapolation of this plot to intercept the abscissa gave a k_i of 0.4 nmol/mg protein (i.e. 0.4 μM). This value is almost equal to that of the dissociation constant of the high affinity site of ∿0.3 μM, suggesting that the high affinity site is also the inhibitor binding site of the OS-ATPase.

Beechey et al. (180) and Cattell et al. (181) have shown that the DCCD binding protein can be extracted from the

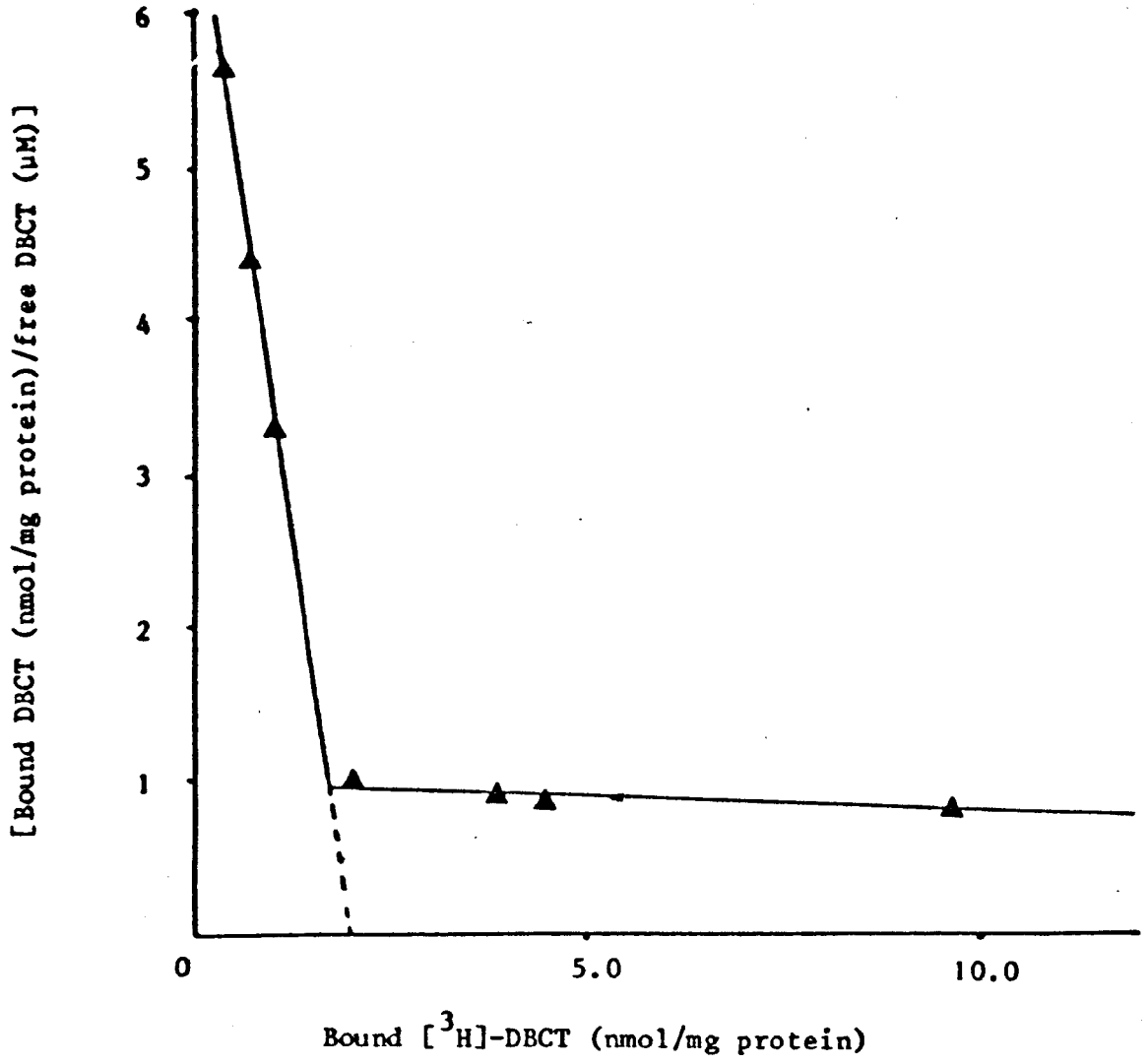


Fig. 3.3 Scatchard plot of [^{3}H]-DBCT binding to beef heart submitochondrial particles

The binding data presented in Fig. 3.1 (▽), were analysed according to Scatchard (176).

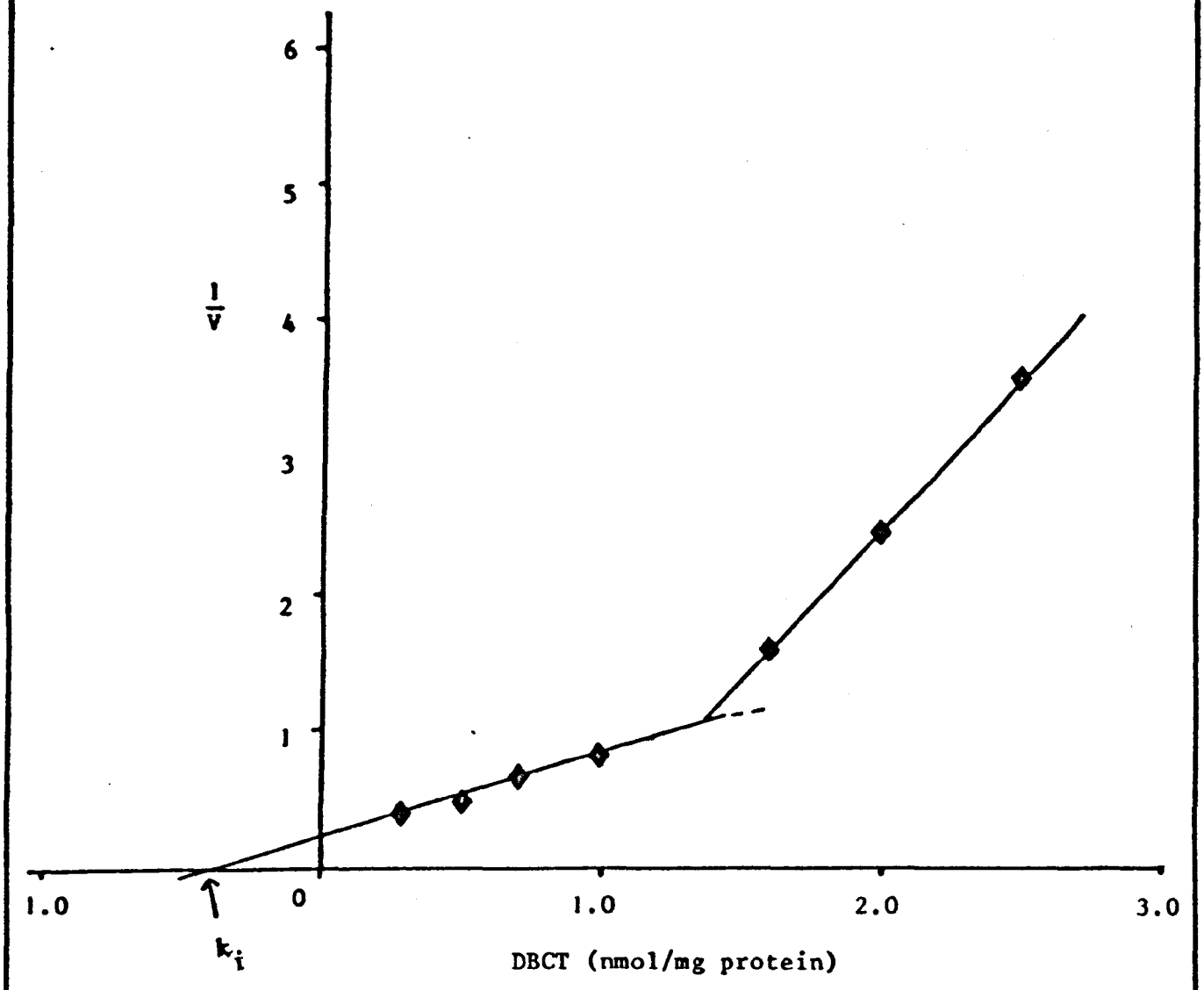


Fig. 3.4 Dixon plot of the inhibition of the ATPase activity of submitochondrial particles by [^{3}H]-DBCT

The ATPase inhibition data presented in Fig. 3.1 (▽), were analysed according to Dixon et al. (179). Each data point is the average of 4 duplicates. The experimental data is presented in the figure as the reciprocal of the specific ATPase activity (1/V) plotted against the total DBCT concentration (nmol/mg protein).

mitochondrial membrane with chloroform-methanol (2:1, v/v). Consequently, an attempt was made to adopt the purification scheme devised by Cattell et al. (181), to isolate the DBCT binding component. The data presented in Table 3.2, shows that the DBCT binding component is readily extracted with chloroform-methanol from [^{3}H]-DBCT 'labelled' submitochondrial particles. In this experiment, more than 87% of the bound [^{3}H]-DBCT was extracted. The removal of extracted membranes from the organic-solvent phase involves filtering through glass wool and this coupled with obvious phase partition effects invariably lead to some loss of extract (∿15-20% of bound DBCT). Washing of the extract with water to remove all the non-proteolipid protein results in a small loss (5%) of radioactivity. The amount of protein removed by the washing procedure appears to be variable and there does not appear to be a correlation between protein removed and radioactivity lost.

The next step in the purification scheme is the ether precipitation step. This step is known to precipitate only protein [Kuntzel et al. (182)] and Cattell et al. (181) have shown that this procedure precipitates the DCCD-binding protein. Table 3.2 shows that 75-80% of the protein remaining after the water wash was precipitated, but only ∿2% of the radioactivity was lost. This result is very different from the DCCD binding data reported by Cattell et al. (181) where 65-70% of the radioactivity is precipitated with 70-80% of the protein. The [^{3}H]-DBCT binding component remained in the ether supernatant.

Thin layer chromatographic analysis of the washed chloroform-methanol extract (Fig. 3.5) shows that the radioactivity is associated with a single spot with an R_f value (∿0.8) in chloroform/methanol/HCl/water solvent system, which is vastly different from that of the free [^{3}H]-DBCT (R_f 0.25). T.l.c. analysis

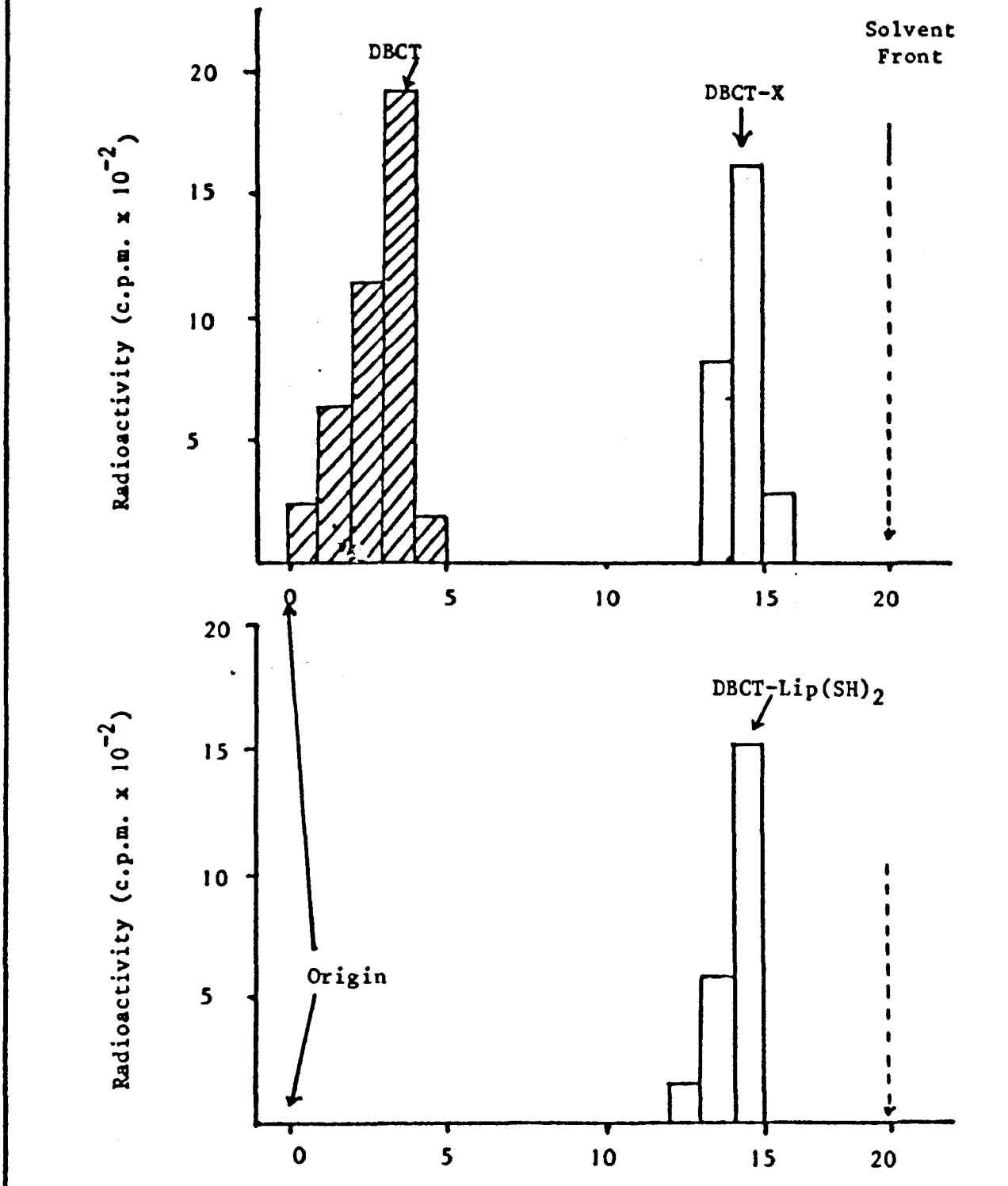


Fig. 3.5 Thin layer chromatography of purified chloroform/methanol extract of DBCT labelled submitochondrial particles

Fig. 3.5 Thin layer chromatography of purified chloroform/methanol extract of DBCT labelled submitochondrial particles

Thin layer chromatographic analysis was carried out on Merck pre-prepared silica-gel TLC plates in a solvent system consisting of chloroform/methanol/HCl/water (650/250/3/36; v/v). Radioactivity was determined by slicing the plates into 0.5 cm x 0.5 cm strips, which were then dropped into a Triton-X100 based scintillation cocktail and counted (as described in Methods) after leaving overnight.

(A) Free [^{3}H]-DBCT and [^{3}H]-DBCT labelled washed chloroform/methanol extract ([^{3}H]-DBCT-X).

(B) [^{3}H]-DBCT-dihydrolipoic acid complex.

Table 3.2 Chloroform/methanol extraction of DBCT binding component

Fraction	Total c.p.m.	Total DBCT (nmol)	% [^{3}H]-DBCT	% Protein
Labelled particles	340,000	660.0	100	100
$CHCl_3/CH_3OH$ extract	296,820	576.2	87.3	18.7
Washed $CHCl_3/CH_3OH$ extract	265,200	514.8	78.0	4.0
Ether precipitate	6,800	13.2	2.0	3.2
Ether supernatant	258,400	501.6	76.0	< 1.0

Submitochondrial particles (66.0 mg) suspended at 10 mg/ml in 0.25 M sucrose, 10 mM tris-Cl, pH 7.5; 1 mM EDTA buffer were preincubated with [^{3}H]-DBCT at 10 nmol/mg protein for 16 hours at 4° C. The labelled particles were extracted for 18 hours with 20 volumes of chloroform/methanol (2:1, v/v). Washing of the extract and precipitation with ether were as described in method. The data presented in the table represents a typical extraction profile of the [^{3}H]-DBCT-X compound.

of the washed extract in two other solvent systems; propan-1-ol or light petroleum (b.p. 60-80° C)/diethyl ether/acetic acid (90:10:1, v/v), also showed only one peak of radioactivity, which was separable from the free [^{3}H]-DBCT and subunit 9.

The labelled component was purified by preparative t.l.c. with two solvent systems (chloroform/methanol/HCl/water, 65:25:0.31:3.6, v/v; and chloroform/methanol/17% (v/v) NH_3, 2:2:1, v/v). The purified component did not exhibit a Folin or ninhydrin reaction. From the above results it may be concluded that DBCT binds to a lipophilic non-protein component of the mitochondrial membrane which is distinct from the DCCD-binding protein (subunit 9).

Preliminary investigation of the [^{3}H]-DBCT labelled component by Cain et al. (149) have indicated that the reactive

component is possibly a dithiol residue of lipoic acid. Fig. 3.5 shows that the DBCT reacts with dihydrolipoic acid to form a complex, which is indistinguishable, (that is, they have the same R_f value) from the labelled component in the three t.l.c. systems mentioned above. However, because these compounds have the same R_f value in the different t.l.c. systems, this does not mean that they are one and the same compound, that is, that the unknown component is lipoic acid. A mass spectral analysis of the purified DBCT-component (Fig. 3.6) does not support the proposal that the unknown component is lipoic acid. Fig. 3.6 shows that the highest mass number obtained is 371, this mass is much lower than that expected for the molecular ion of the DBCT-lipoic acid complex of 489 (Fig. 3.7). From the mass spectral analysis, the estimated molecular weight of the unknown component X is 97-112. Fig. 3.8 shows the mass spectra of lipoic acid (A) and DBCT (B). Fig. 3.8B indicates a molecular ion of lipoic acid of 206, and that further breakdown of the molecule into smaller molecular ions occurs initially with the loss of the sulphur atoms.

3.5 DISCUSSION

The results presented in this Chapter have shown that two types of binding sites (high affinity and low affinity binding sites) for DBCT is present on beef heart submitochondrial particles. The maximum number of high affinity sites is $\sim$2.0 nmol/mg protein and the apparent dissociation constant (k_D) of these sites is 0.286 µM (Fig. 3.3). The estimated number of low affinity sites on submitochondrial particles is 33.0 nmol/mg protein, having apparent dissociation constant of 30.5 µM. The high affinity sites thus account for less

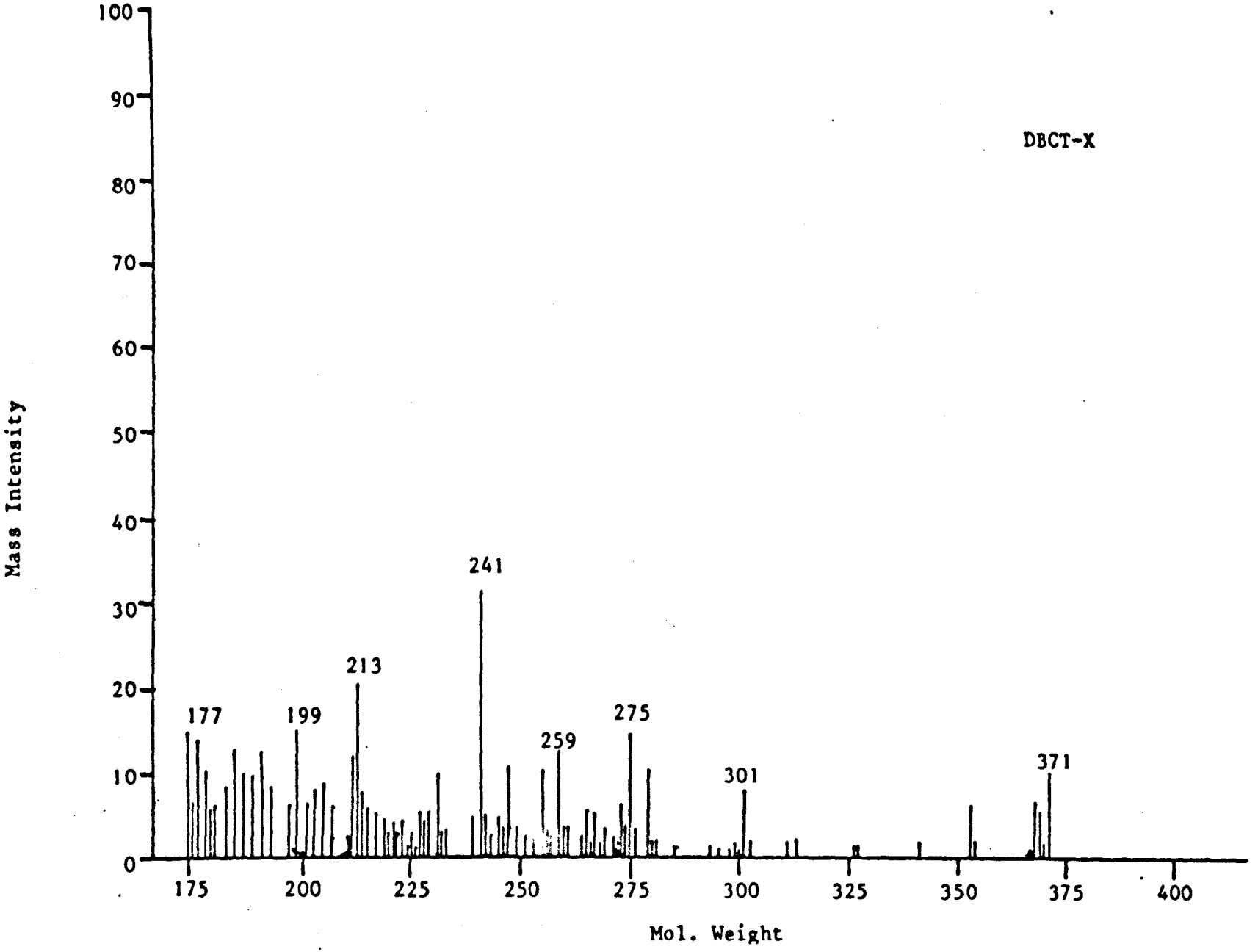


Fig. 3.6 Mass spectra of DBCT-X

The figure shows mass intensity report of DBCT-X isolated from submitochondrial particles. DBCT-X was analysed as a solution in chloroform (see Methods).

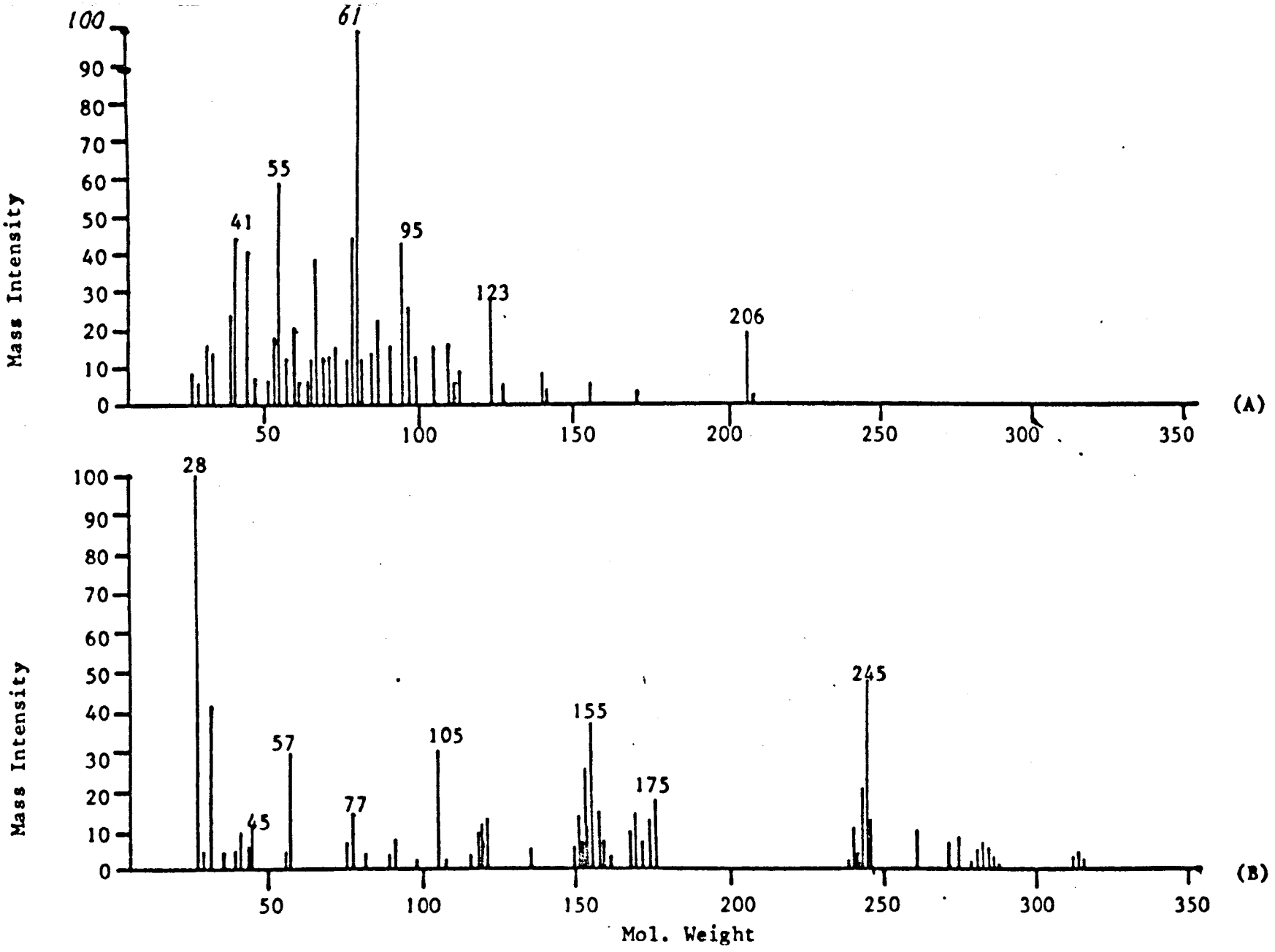


Fig. 3.7 Mass spectra of lipoic acid (A) and DBCT (B)

The figure shows a mass intensity report of lipoic acid (A) and DBCT (B), analysed as a chloroform solution as described in Methods.

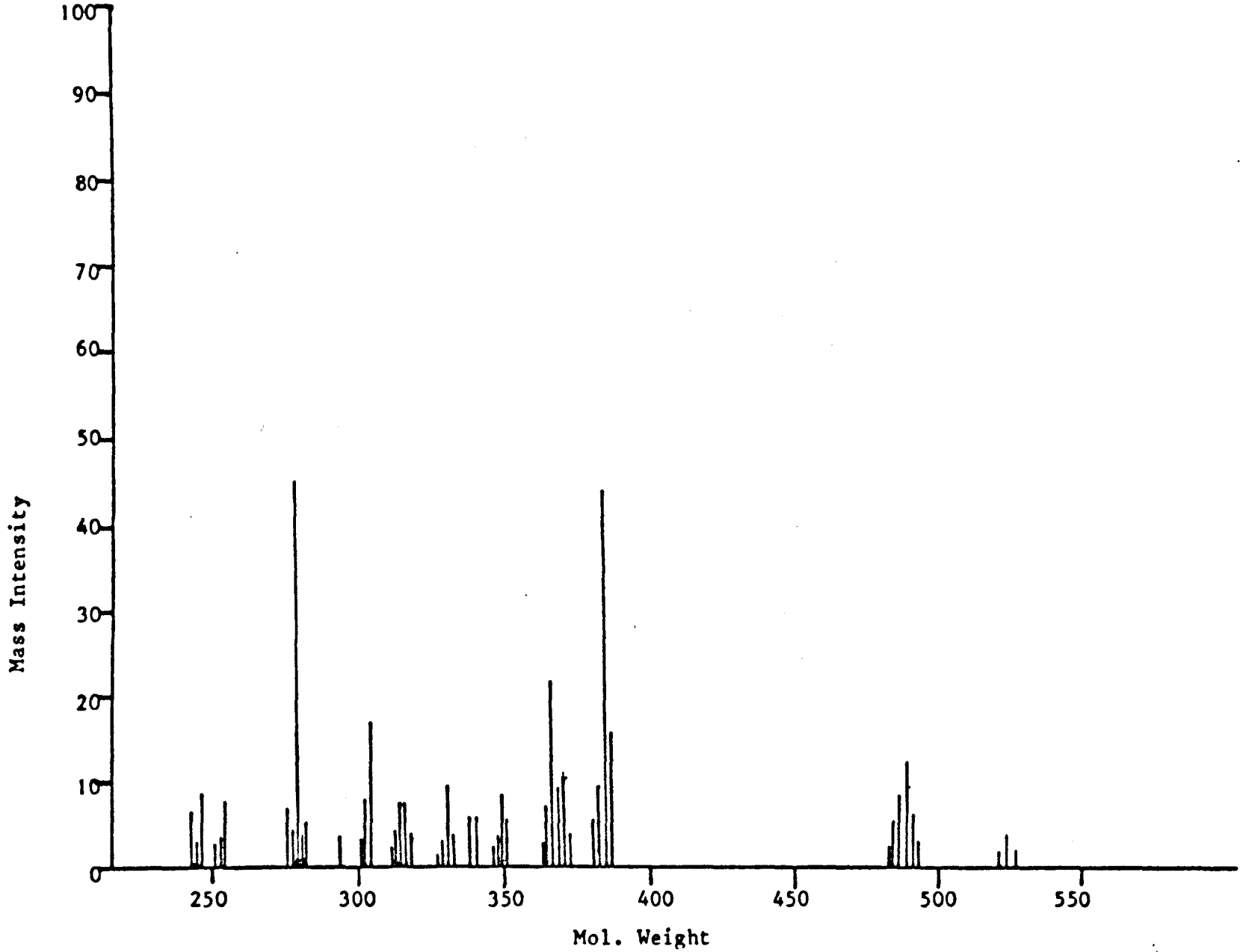


Fig. 3.8 Mass spectra of DBCT-lipoic acid complex

The figure shows a mass intensity report of DBCT-lipoate complex, analysed as in Fig. 3.7.

than 6% of the total number of binding sites present, and binds DBCT 100 times more tightly than the low affinity sites. The apparent dissociation constant of the high affinity site ($k_D \sim 0.3$ μM) almost equal to the inhibitor dissociation constant (k_i) of 0.4 nmol/mg protein (∿0.4 μM) for DBCT inhibition of the ATPase activity of submitochondrial particles (Fig. 3.4). These values ($k_D \sim 0.3$ μM, $k_i \sim 0.4$ μM) are in good agreement with the concentration of DBCT required for inhibition of the ATPase activity (Fig. 3.1), but not with the concentration of DBCT required for inhibition of oxidative phosphorylation in submitochondrial particles (see Chapter 2). The maximum number of high affinity binding sites (∿2.0 nmol/mg protein) is almost equal to the amount of DBCT bound (1.9 nmol/mg protein) at 90% inhibition of the ATPase activity (Fig. 3.1). These results thus show that ATPase inhibition is correlated with the saturation of the high affinity binding sites.

Contrary to Cain et al. (149) and Aldridge et al. (143) we believe that binding to the low affinity sites and not binding to the high affinity sites is responsible for inhibition of oxidative phosphorylation. This conclusion is drawn from the fact that, at DBCT concentration (2-5 nmol/mg protein) causing saturation of the high affinity site and total inhibition of the ATPase activity (Fig. 3.1), oxidative phosphorylation in submitochondrial particles is not significantly affected (see Chapter 2, Fig. 2.6). These results thus support the proposal in Chapter 2 that trialkyl tin inhibition of oxidative phosphorylation and ATP hydrolysis (ATP dependent reactions) occur by two different mechanisms, and re-enforce the proposal of Pedersen (81) that the OS-ATPase complex is at least bifunctional.

Although Cain et al. (149) have reported that the high affinity sites for DBCT and other trialkyl tins were located in

the OS-ATPase complex, DBCT was not found to bind to any of the protein component of the complex and the fact that the high affinity sites for DBCT are not competed for by tributyl tin, dibutyl tin dichloride or the penta-coordinate compound Ve_{2283}, indicating that DBCT binds at different sites from these organotin compounds. That DBCT does not bind to a protein component of the ATPase was shown by the fact that, unlike bound [^{14}C]-DCCD, all the bound [^{3}H]-DBCT can be removed by extraction with 10% water in acetone (Fig. 3.1). Extraction of the [^{3}H]-DBCT component complex by the method of Cattell et al (181) demonstrated that DBCT does not bind to subunit 9, the DCCD binding protein. These results thus suggest that DBCT binds a non-protein hydrophobic molecule.

The nature of the DBCT binding component is still unknown. However, from preliminary studies Cain et al. (149) have suggested that it could be lipoic acid or one of its protein conjugated forms. However, extraction of the DBCT-X (binding component-X) followed: by its purification by preparative thin layer chromatography; the compound gave netative Folin and ninhydrin test for protein. Although the compound had similar R_f values to authentic DBCT-lipoic acid in the solvent system tested, (Fig. 3.5) mass spectral analysis of DBCT-X (Fig. 3.6) and DBCT-lipoic acid indicates that they were not the same compound (i.e. X is not lipoic acid).

CHAPTER 4

4. THE ROLE OF DIHYDROLIPOIC ACID IN OXIDATIVE PHOSPHORYLATION

4.1 INTRODUCTION

The ability of dihydrolipoic acid to drive ATP synthesis and stimulate energy-linked reactions in beef heart mitochondria has been reported by Griffiths (132) and Mitchell(243). However, no satisfactory explanation has been put forward to account for the actual role played by dihydrolipoic acid during ATP synthesis. Whilst Griffiths (132) has proposed a chemical coupling intermediate role for dihydrolipoic acid, D. Mitchell (243) has suggested that dihydrolipoic acid acts as a trans-membrane proton translocator.

The findings that trialkyl tin inhibition of oxidative phosphorylation and other energy-linked reactions could be reversed by dihydrolipoic acid, combined with its ability to drive oligomycin-sensitive ATP synthesis in mitochondria, led Griffiths (132) to suggest that dihydrolipoic acid might act as the high-energy intermediate proposed by Slater (14) and Sanadi et al. (244) in their chemical coupling hypothesis. Dihydrolipoic acid would then provide the link between the respiratory chain and the ATP synthase complex which catalyse the terminal reactions of oxidative phosphorylation. These terminal reactions, outlined in Fig. 4.1, are proposed by Griffiths to involve a cycle of transacylation and transphosphorylation reactions analogous to those involved in the succinic thiokinase system, in substrate level phosphorylation. The reaction sequence proposed by Nishimura and Grinnell (245) for the succinic thiokinase is shown below (equations 1-4).

1. Succinyl-S-lipoate + CoASH $\rightleftharpoons$ Succinyl-S-CoA + lipoate
2. Succinyl-S-CoA + Enz $\rightleftharpoons$ CoA-S-Enz + succinate
3. CoA-S-Enz + Pi $\rightleftharpoons$ P$\sim$Enz + CoASH
4. P$\sim$Enz + GDP $\rightleftharpoons$ GTP + Enz

Enz is the enzyme and P$\sim$Enz its phosphorylated complex.
'$\sim$' represents the high energy bond.

The proposals of Griffiths, thus suggest that dihydrolipoic acid was directly involved in the generation of ATP, and that the respiratory chain was not required for dihydrolipoic acid driven ATP synthesis in mitochondria. Support for Griffiths hypothesis is provided by Bertoli et al. (246) who has shown that yeast promitochondria lacking a functional electron transport system due to lack of cytochromes and ubiquinones (247, 248) but possessing a functional energy transfer system, was able to catalyse dihydrolipoic acid, oleoyl-lipoate and oleoyl-phosphate driven ATP synthesis.

Mitchell (243), contrary to Griffiths, has suggested that dihydrolipoic acid was indirectly involved in ATP synthesis. Mitchell (243) proposed that dihydrolipoic acid was not a high-energy chemical intermediate of oxidative phosphorylation, but that it acts as a transmembrane proton translocator used in building up the transmembrane potential gradient used to drive ATP synthesis and other energy-linked reactions. Unlike Griffiths, he claimed that a functional respiratory chain was required, since electron transport and proton transport were directly interdependent. The proposals of Mitchell can thus be represented diagramatically as:

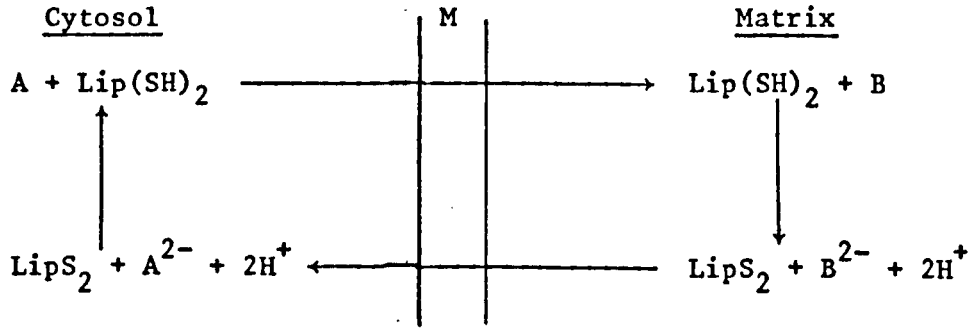


Fig. 4.1 Transmembrane transport of protons by dihydrolipoic acid

M = mitochondria inner membrane; A and B are components of the respiratory chain; $Lip(SH)_2$, dihydrolipoic acid and $LipS_2$, lipoic acid.

The resulting transmembrane proton potential is thus used to drive ATP synthesis as postulated in the chemiosmotic hypothesis.

In this Chapter we examine the ability of dihydrolipoic acid, oleoyl-lipoate and oleoyl-phosphate to drive ATP synthesis and some energy-linked reaction catalysed by beef heart mitochondria, submitochondrial particles and complex V. We try to provide an explanation for the role played by these compounds and their significance in mitochondrial energy-transfer processes.

4.2 MATERIALS

All reagents used were of AnalaR grade. AnalaR grade organic solvents such as methanol and chloroform were redistilled before use. Dihydrolipoic acid, oleic acid, oleoyl chloride, CoASH and acyl-SCoA were obtained from Sigma Chemical Company. NAD, NADP, ATP, ADP and 5,5-dithiobis(2-nitrobenzoic) acid were obtained as previously stated in the Materials sections of Chapter 2.

4.3 METHODS

Oxidative phosphorylation and ATP hydrolysis ATP-driven and succinate driven transhydrogenation were assayed as described in the Methods section of Chapter 2. The method of Ellman (158) was used to measure the concentration of thiol groups.

Preparation of oleoyl-S-lipoate

Dihydrolipoic acid (0.5 mmole) was dissolved in 10 ml redistilled tetrahydrofuran + 10 ml triple distilled water. The pH was adjusted to 7.5 with 1 M NaOH. Oleoyl chloride (0.5 mmole) was then added to this reaction mixture with rapid stirring. The mixture was then stirred in the dark at room temperature for 3-4 hours; the

pH was monitored and kept between pH 7.5-8.0 by addition of 1 M NaOH. The mixture was assayed for thiol content by the DTNB method of Ellman (158). When the reaction was adjusted to be complete (using the disappearance of thiol as the parameter), the reaction mixture was acidified to pH 2-3 by addition of 1 M HCl and extracted with 4 5 vol. of diethyl ether which was then removed from the reaction product by rotary evaporation. Oleoyl-S-lipoate was the major reaction product accounting for 75 % of the final product. The crude oleoyl-S-lipoate was purified using preparative thin layer chromatography in a solvent system containing toluene; ethyl formate and formic acid (5:4:1, v/v). Oleoyl-S-lipoate was dissolved in dimethylformamide and assayed by the hydroxamate method of Lipmann and Tuttle (249).

Oleoyl-phosphate preparation

Oleoyl phosphate was prepared from anhydrous phosphoric acid and oleoyl chloride as described by Griffiths (132). Dry orthophosphoric acid (0.325 g) was weighed into a round bottomed, quickfit, 250 ml flask, to which was added 30 ml dry, redistilled acetonitrile. The contents were then stirred using a magnetic stirrer and follower. Triethylamine (500 µl) was added to facilitate the dissolution of the orthophosphoric acid. Oleoyl chloride (1 ml) was then added, and the reaction was allowed to proceed for 1½ hours in the dark at room temperature. The reaction was stopped by addition of 100 ml 1 M sodium chloride, followed by the addition of 70 ml redistilled chloroform. The reaction mixture was shaken and transferred to a separating funnel where the chloroform and aqueous phases were allowed to separate. The chloroform phase was isolated and the aqueous phase re-extracted with 30 ml chloroform and the chloroform phases were pooled. This was then shaken with water to remove any free phosphoric acid and after separation the chloroform

layer was dried over anhydrous sodium sulphate for 10-20 minutes. The chloroform layer was decanted from the sodium sulphate and filtered through chloroform washed Whatman No 1 filter to remove any suspended sodium sulphate. The filtrate was then evaporated to dryness on a rotary evaporator. The residue is crude oleoyl-phosphate. Thin layer chromatography in a chloroform/methanol (2:1, v/v) solvent system shows it to consist of one spot. Hydroxamate assay of this residue shows that it was 90-95% pure oleoyl-phosphate.

Hydroxamate assay for acyl esters (249)

Tubes containing the acyl ester under test and 100 µl 10% sodium dodecyl sulphate (electrophoretic grade) were made up to a final volume of 0.5 ml with distilled water. 0.5 ml of hydroxylamine reagent [28% hydroxylamine-HCl and 14% NaOH, (w/v) and 0.5 ml acetate buffer 0.1 M acetic acid, 0.1 sodium acetate mixed 2:8, (v/v)] were then added, the mixture vortexed and allowed to stand for 10 minutes at room temperature. 0.5 ml 36% HCl, 0.5 ml 12% trichloroacetic acid and 0.5 ml of ferric chloride reagent (6% $FeCl_3.6H_2O$ in 0.1 M HCl) were added with mixing. The mixtures were allowed to stand for 15 minutes at room temperature and their optical density at 490 nm was measured in a Unicam SP1800 spectrophotometer. The amount of acyl ester was then estimated from a standard curve constructed using acetyl phosphate, or methyl oleate as standard..

4.4 RESULTS

The results presented in Table 4.1, depict the ability of dihydrolipoic acid to drive ATP synthesis in beef heart mitochondria in the presence or absence of the respiratory inhibitors; rotenone and antimycin A. The results show that ATP synthesis driven by

Table 4.1 Dihydrolipoic acid driven ATP Synthesis in Beef Heart Mitochondria

Additions	ATP synthesised nmol/min/mg protein
None	0.0
$Lip(SH)_2$ (1 µmol)	43.0 ± 15.0
$Lip(SH)_2$ (2 µmol)	70.0 ± 25
α-Lipoic acid (1 µmol)	0.0
Pyruvate/malate	192.5 ± 10
Succinate (20 µmol)	135.0 ± 10
Ascorbate/TMPD	94.0 ± 20
$Lip(SH)_2$ + Rotenone (2 µg) + antimycin A (2 µg)	0.0
Succinate (20 µmol) + rotenone (2 µg) + antimycin A (2 µg)	0.0
Pyruvate/malate + rotenone (2 µg) + antimycin A (2 µg)	0.0
Asborbate/TMPD + rotenone (2 µg) + antimycin A (2 µg)	89.0 ± 20
$Lip(SH)_2$ + rotenone (2 µg)	0.0
$Lip(SH)_2$ + antimycin A (2 µg)	0.0

ATP synthesis was assayed in a 'glucose-hexokinase trap' system as described in Materials and Methods. Mitochondria (2 mg) were preincubated in phosphorylation medium ± rotenone (2 µg) and antimycin A (2 µg), for 5 minutes at 30° C. The reaction was then initiated by addition of the appropriate substrate. Each value represents the mean of five duplicates.

dihydrolipoic acid in beef heart mitochondria, only occurs in the absence of rotenone and antimycin A. The rate of ATP synthesis was found to be dependent upon the concentration of dihydrolipoic acid, and to be much slower than the rates of pyruvate/malate, succinate or ascorbate/TMPD driven ATP synthesis (Table 4.1). This difference in rate is due primarily to the low concentration of dihydrolipoic acid (2 mM) (compared with 20 mM pyruvate or 20 mM succinate) used in the assay. The use of low concentration of dihydrolipoic acid is unavoidable since higher concentration (4-5 mM) inhibits (uncouples) ATP synthesis. In the presence of rotenone and/or antimycin A dihydrolipoic acid driven ATP synthesis in beef heart mitochondria is completely inhibited (Table 4.1). Table 4.2 shows that dihydrolipoic acid alone was ineffective in driving ATP synthesis in submitochondrial particles, either in the presence or absence of rotenone and antimycin A.

The results presented in Tables 4.1 and 4.2, thus suggest that oxidation of dihydrolipoic acid via, the respiratory chain is necessary for ATP synthesis driven by dihydrolipoic acid in mitochondria. This is supported by the fact that α-lipoic acid does not drive ATP synthesis in mitochondria or submitochondrial particles. The finding (Table 4.1) that rotenone and/or antimycin A alone is able to inhibit dihydrolipoic acid driven ATP synthesis in mitochondria, indicates that the electrons deriving from dihydrolipoic acid enters the respiratory chain at site I (Fig. 4.2). However, the fact that ATP synthesis does not occur in submitochondrial particles actively synthesising ATP driven by succinate (Table 4.2), suggests that the electron deriving from dihydrolipoic acid does not enter the respiratory chain directly, but through an intermediate electron carrier.

The results presented in Tables 4.3 and 4.4 illustrate

Table 4.2 ATP synthesis in submitochondrial particles

Additions	ATP synthesised nmol/min/mg protein
None	0.0
$Lip(SH)_2$ (1 µmol)	0.0
$Lip(SH)_2$ (2 µmol)	0.0
α-Lipoic acid (1 µmol)	0.0
Succinate (20 µmol)	90.0
$Lip(SH)_2$ (2 µmol) + Rotenone (2 µg) + antimycin A	0.0
Succinate (20 µmol) + Rotenone (2 µg) + antimycin A	0.0
$Lip(SH)_2$ (1 µmol) + Succinate (20 µmol)	85.0
$Lip(SH)_2$ (1 µmol) + succinate (2 µmol) + rotenone (2 µg) + antimycin A (2 µg)	0.0

ATP synthesis was assayed as described in Table 4.1 except that submitochondrial particles (2 mg) were used instead of mitochondria. Each value is the mean ± s.d. of four duplicates. Amount of each component added is indicated in brackets. $Lip(SH)_2$, dihydrolipoic acid.

that ATP synthesis in mitochondria and submitochondrial particles is concomitant with the oxidation of dihydrolipoic acid. The results show that in the presence of rotenone and/or antimycin A, no oxidation of dihydrolipoic acid (monitored using Ellman's reagent DTNB) or synthesis of ATP occur in mitochondria (Table 4.3). However, in the absence of rotenone and antimycin A, oxidation of dihydrolipoic acid, and synthesis of ATP occurs simultaneously, and under the conditions of the experiment a Pi/dihydrolipoic acid ratio of 2.4 ± 0.15 was obtained. This ratio was found to be fairly

Table 4.3 Dihydrolipoic acid ATP synthesis in mitochondia

Additions	Δ µmol $Lip(SH)_2$ (oxidised)	Δ µmol Pi (uptake)	Pi/Lipoic acid
Experiment A			
None			
$Lip(SH)_2$ (1 µmol)	0.78	0.96	1.2 ± 0.0
$Lip(SH)_2$ (2 µmol)	1.4	3.90	2.8 ± 0.2
$Lip(SH)_2$ (1 µmol) + rotenone + antimycin A	0.0	0.0	0.0
$Lip(SH)_2$ (2 µmol) + rotenone + antimycin A	0.0	0.0	0.0
$Lip(SH)_2$ (2 µmol) + rotenone (2 µg)	0.0	0.0	0.0
$Lip(SH)_2$ (2 µmol) + antimycin A (1 µg)	0.0	0.0	0.0
Experiment B			
$Lip(SH)_2$ (2 µmol) 20 min incubation	1.12 ± 0.0	2.85 ± 0.0	2.54 ± 0.1
$Lip(SH)_2$ (2 µmol) 40 min incubation	1.40 ± 0.0	3.90 ± 0.0	2.80 ± 0.0
$Lip(SH)_2$ (2 µmol) 60 min incubation	1.56 ± 0.0	4.1 ± 0.0	2.63 ± 0.0
$Lip(SH)_2$ (2 µmol) 80 min incubation	1.89 ± 0.0	4.3 ± 0.0	2.0 ± 0.0

ATP synthesis was assayed by Pi uptake as described in Fig. 4.1. Oxidation of dihydrolipoic acid was monitored by measuring the disappearance of free thiol groups using the method of Ellmann (158). Each value is the mean of 5 duplicates. $Lip(SH)_2$ dihydrolipoic acid.

independent of the time of incubation, but varies appreciably from one mitochondrial preparation to another (1.8-2.8). Table 4.4 shows that oxidation of dihydrolipoic acid and synthesis of ATP by submitochondrial particles only occurs when the cofactor NAD^+ (nicotinamide adenine dinucleotide) is added to the assay system. The efficiency of conversion of dihydrolipoic acid to ATP is very low giving a Pi/dihydrolipoic acid ratio of less than 1 (Table 4.4). As in the case of mitochondria, dihydrolipoic acid oxidation, and the concomitant ATP synthesis is completelyinhibited by rotenone and antimycin A (Table 4.4). The results presented in Tables 4.3 and 4.4 thus confirm the suggestion that the electrons deriving from dihydrolipoic acid enter the respiratory chain at site I, and indicate that the intermediate electron carrier was NAD^+.

Dihydrolipoic acid driven ATP synthesis in mitochondria is sensitive to inhibitors (oligomycin, DCCD) and uncouplers (TTFB, '1799' valinomycin) of oxidative phosphorylation (Table 4.5). The data presented in Table 4.5 shows that in the absence of the respiratory inhibitors, rotenone and antimycin A, the uncouplers and ionophores examined cause complete oxidation of dihydrolipoic acid without concomitant synthesis of ATP. Oligomycin and DCCD, unlike the uncouplers, not only cause complete inhibition of ATP synthesis, but also inhibit the rate of oxidation of dihydrolipoic acid in mitochondria. In the presence of oligomycin or DCCD 25% and 50% of the dihydrolipoic acid was oxidised, respectively. However, the ability of oligomycin to inhibit dihydrolipoic acid oxidation was dependent upon the mitochondrial preparation. Addition of the uncoupler TTFB to oligomycin treated mitochondria, causes complete oxidation of dihydrolipoic acid (Table 4.5). The results thus demonstrate that a typical energy transfer system is functioning during dihydrolipoic acid driven ATP synthesis, similar to that

Table 4.4 Dihydrolipoic acid driven ATP synthesis in submitochondrial particles

Additions	$Lip(SH)_2$ oxidised (µmol)	Pi uptake (µmol)	Pi/lipoic acid
$Lip(SH)_2$ (1 µmol)	0.0	0.0	0.0
$Lip(SH)_2$ (2 µmol)	0.0	0.0	0.0
$Lip(SH)_2$ (1 µmol) + NAD^+ (50 nmol)	1.0	0.30	0.30
$Lip(SH)_2$ (2 µmol) + NAD^+ (50 nmol)	2.0	1.18	0.59
Succinate	-	5.1	-
$Lip(SH)_2$ (2 µmol) + rotenone (2 µg) + antimycin A (2 µg)	0.0	0.0	0.0
$Lip(SH)_2$ (2 µmol) + NAD^+ + rotenone (2 µg) + antimycin A	0.0	0.0	0.0

ATP synthesis and dihydrolipoic acid oxidation were assayed as described in Table 4.5. Submitochondrial particles (2 mg) were preincubated in phosphorylation medium [± rotenone (2 µg) antimycin A (2 µg) ± NAD^+ (50 nmol)] for 5 minutes at 30° C. The reaction was initiated by addition of dihydrolipoic acid (1 or 2 µmol) and allowed to run for 40 minutes. Total volume 1.1 ml. Each value is the mean of five duplicates.

Table 4.5 Inhibitor sensitivity of dihydrolipoic acid driven ATP synthesis in mitochondria

Additions	(DHL) oxidised µmol	ATP synthesised µmol Pi
Dihydrolipoic acid (2 µmol)	1.83 ± 0.47	4.46 ± 1.2
" + DCCD	0.98 ± 0.3	0.0
" + oligomycin	0.54 ± 0.40	0.0
" + TTFB	2.00	0.0
" + CCCP	2.00	0.0
" + '1799'	2.00	0.0
" + gramicidin	2.00	0.0
" + valinomycin	2.00	0.0
" + 2,4-DNP	2.00	0.0
" + oligomycin	2.00	0.0
Succinate (20 µmol)	-	4.86

ATP synthesis and dihydrolipoic acid oxidation were assayed as described in Table 4.3. Mitochondria (2 mg) were incubated in phosphorylation buffer ± the inhibitor indicated above (2 µg each) for 5 minutes at 30° C. The reaction was initiated by addition of dihydrolipoic acid (2 µmol) or succinate and allowed to proceed for 20 minutes. Total volume 1.1 ml. Samples were removed for estimation of inorganic phosphate and free thiols. Each value is the mean of five duplicates.

obtained when pyruvate/malate or succinate is used as respiratory substrate.

When dihydrolipoic acid was replaced by oleoyl-lipoate as substrate, no ATP synthesis was obtained either in mitochondria (Table 4.6), submitochondrial particles (Table 4.7) or complex V (Table 4.8), either in the presence or absence of rotenone and antimycin A. Addition of catalytic amounts (10-20 nmol) of oleoyl-phosphate, oleoyl-SCoA and/or oleic acid did not stimulate oleoyl-

Table 4.6 ATP synthesis in mitochondria driven by various substrates

Additions	Total Pi uptake (μmol/20 min)	ATP synthesised nmol/min/mg
Experiment A		
None		
$Lip(SH)_2$ (2 μmol)	1.86	93.0
Pyruvate/malate (20/2 μmol)	3.4	170.0
Succinate (20 μmol)	2.7	135.0
Oleoyl-lipoate (1 μmol)	0.0	0.0
Oleoyl-lipoate (2 μmol)	0.0	0.0
Experiment B (+ rotenone + antimycin A)		
None	0.0	0.0
$Lip(SH)_2$(2 μmol)	0.0	0.0
Oleoyl-lipoate (1 μmol)	0.0	0.0
Oleoyl-lipoate (2 μmol)	0.0	0.0
Experiment C		
Succinate + oleoyl-phosphate (1 μmol)	0.0	0.0
Succinate + oleoyl-lipoate (1 μmol)	0.0	0.0
Pyruvate/malate + oleoyl-lipoate (1 μmol)	0.0	0.0
$Lip(SH)_2$ + oleoyl-lipoate (1 μmol)	0.0	0.0
$Lip(SH)_2$ + oleoyl-phosphate (1 μmol)	0.0	0.0

ATP synthesis was assayed as described in Table 4.1. Mitochondria (1 mg) ± rotenone (rot) and antimycin A (2 μg each) were preincubated in phosphorylation at 30° C for 5 minutes. The appropriate substrates were then added to initiate the reaction. Final volume 1.1 ml. Each value is the mean of four duplicates.

Table 4.7 Ability of various substrates to drive ATP synthesis catalysed by submitochondrial particles

Additions	ATP synthesis/20 min/mg	
	µmol Pi	µmol G6P
Experiment A		
None	0.0	0.0
$Lip(SH)_2$ (2 µmol)	0.0	0.0
Oleoyl-lipoate (1 µmol)	0.0	0.0
Oleoyl-lipoate (2 µmol)	0.0	0.0
Oleoyl-phosphate (1 µmol)	0.0	0.0
Oleoyl-phosphate (2 µmol)	0.0	0.0
Succinate (20 µmol)	2.7	2.2
Succinate + oleoyl-lipoate (1 µmol)	0.0	0.0
Succinate + oleoyl-phosphate (1 µmol)	0.0	0.0
Experiment B (+ rotenone + antimycin A)		
$Lip(SH)_2$ + oleoyl-lipoate (10 nmol)	0.0	0.0
$Lip(SH)_2$ + oleoyl-CoA (10 nmol)	0.0	0.0
$Lip(SH)_2$ + oleoyl-phosphate (10 nmol)	0.0	0.0
Succinate	0.0	0.0

ATP synthesis was estimated in a glucose-hexokinase trap system as described in Table 4.1. Submitochondrial particles (2 mg) were preincubated in phosphorylation medium containing rotenone (2 µg) and antimycin A, for 5 minutes at 30° C. The reaction was initiated by the addition of the appropriate substrates as indicated in the Table. The reaction was allowed to proceed for 20 minutes. Samples were then removed for estimation of inorganic phosphate and glucose-6-phosphate. Each value is the mean of four duplicates.

Table 4.8 ATP synthesis catalysed by complex V

Additions	ATP synthesised nmol/min/mg
$Lip(SH)_2$ (2 µmol)	0.0
Oleoyl-lipoate (1 µmol)	0.0
Oleoyl-lipoate (2 µmol)	0.0
Oleoyl-phosphate (1 µmol)	0.0
Oleoyl-phosphate (2 µmol)	0.0
$Lip(SH)_2$ + oleic acid (10 nmol)	0.0
$Lip(SH)_2$ + oleoyl-CoA (10 nmol)	0.0
Oleoyl-lipoate + CoASH (10 nmol) + $Lip(SH)_2$	0.0
Oleoyl-phosphate (10 nmol) + CoASH (10 nmol) + $Lip(SH)_2$	0.0

ATP synthesis was estimated in a glucose-hexokinase trap system as described in Materials and Methods. Complex V (0.5 mg) prepared by the method of Hatefi (151) was preincubated in phosphorylation medium for 5 minutes at 30° C. Total volume 1.0 ml. The reaction was initiated by the addition of the appropriate substrate as indicated in the Table. The reaction was allowed to proceed for 20 minutes and samples removed for estimation of glucose-6-phosphate. Glucose-6-phosphate was assayed as described in Materials and Methods. Each value is the mean of five duplicates.

lipoate driven ATP synthesis in submitochondrial particles (Table 4.7) or complex V (Table 4.8). Although catalytic amounts (10 nmol) of oleoyl-lipoate did not affect succinate driven ATP synthesis in mitochondria or submitochondrial particles, a substrate amount (1 µmol) of oleoyl-lipoate was found to inhibit succinate driven ATP synthesis completely (Tables 4.6 and 4.7). The inhibition of succinate driven ATP synthesis by oleoyl-lipoate and oleoyl-phosphate is probably due to their 'detergent-like' properties at such high concentrations (1 mM). This caused disruption of the mitochondrial

membrane structure and results in uncoupling of oxidative phosphorylation. Similar results to those presented in Tables 4.6-4.8 were obtained recently by Dickinson (250), Beauclerk (223) and Solaini (173) working on the same systems. These results are not surprising, since most fatty acids and their acyl-phosphate esters have been shown to be potent uncouplers of oxidative phosphorylation, and energy-linked reactions in mitochondria, and submitochondrial particles (see Chapter 5).

4.5 EFFECT OF DIHYDROLIPOIC ACID AND OLEOYL-LIPOATE ON NADH/NADP TRANSHYDROGENASE REACTION OF BEEF HEART SUBMITOCHONDRIAL PARTICLES

The ability of dihydrolipoic acid and oleoyl-lipoate to drive the energy-linked NADH/$NADP^+$ transhydrogenase reaction in beef heart submitochondrial particles was investigated. The results presented in Table 4.9 show that neither dihydrolipoic acid, oleoyl-lipoate nor oleoyl-phosphate was able to drive the NADH/$NADP^+$ transhydrogenase reaction in beef heart submitochondrial particles, having very active ATP and succinate driven transhydrogenase activity. This experiment was repeated on over sixty different preparations of beef heart submitochondrial particles, and on no occasion was dihydrolipoic acid or oleoyl-lipoate found to stimulate or drive transhydrogenation. Although dihydrolipoic acid driven transhydrogenase activity in submitochondrial particles has been reported by M. Carver (240), it must be emphasised that the reported frequency of occurrence was less than once in every hundred cases. This points to experimental error on his part, or an artefactual reaction. However, Carver has suggested that dihydrolipoic acid was not the high energy chemical intermediate,

Table 4.9 Effect of dihydrolipoic acid and certain acyl-esters on energy-linked transhydrogenase activity in submitochondrial particles

Additions	Transhydrogenase activity (nmol $NADPH_f$/min/mg)
None	0.0
$Lip(SH)_2$ (2 µmol)	0.0
Oleoyl-lipoate (1 µmol)	0.0
Oleoyl-phosphate (1 µmol)	0.0
$Lip(SH)_2$ + oleic acid (10 nmol), oleol-CoA (10 nmol)	0.0
Oleoyl-lipoate (1 mol) + oleoyl-CoA (10 nmol) oleoyl-phosphate (10 nmol)	0.0
ATP	48.0
ATP + oleic acid (1 µmol)	0.0
ATP + oleoyl-phosphate (1 µmol)	0.0
ATP + oleoyl-lipoate (1 µmol)	0.0
Succinate (50 µmol)	62.0
Succinate + oleic acid (1 µmol)	50.0
Succinate + oleoyl-phosphate (1 µmol)	58.0
Succinate + oleoyl-lipoate (1 µmol)	60.0

Transhydrogenase activity was assayed as described in Materials and Methods. Submitochondrial particles (1 mg) were preincubated in transhydrogenase buffer containing 0.25 M sucrose, 50 mM tris-HCl, pH 8.0 (± oleic acid, oleoyl-CoA, oleoyl-phosphate) for 5 minutes at 20° C. The reaction was initiated by the addition of the appropriate substrate, as indicated in the Table. Each value is the mean of four duplicates.

and that the intermediate responsible for driving the transhydrogenase activity might be one of the many reaction products of dihydrolipoic acid.

The data presented in Table 4.9 also show that addition of catalytic amounts (10 nmol) of either oleoyl-CoA, oleoyl-phosphate or oleic acid did not stimulate a dihydrolipoic acid or oleoyl-lipoate driven transhydrogenase activity. Although catalytic amounts (10 nmol) of oleic acid, oleoyl-phosphate and oleoyl-lipoate did not affect ATP driven transhydrogenase activity, substrate amounts (1 μmol) of these compounds completely abolished the reaction. However, succinate driven transhydrogenase activity was not significantly affected by 1 μmol oleic acid, oleoyl-phosphate or oleoyl-lipoate (Table 4.9). These results suggest that the oleoyl-lipoate, oleoyl-phosphate, and oleic acid uncouples ATP-driven transhydrogenase (cf. oxidative phosphorylation), but does not uncouple the respiratory chain linked reaction.

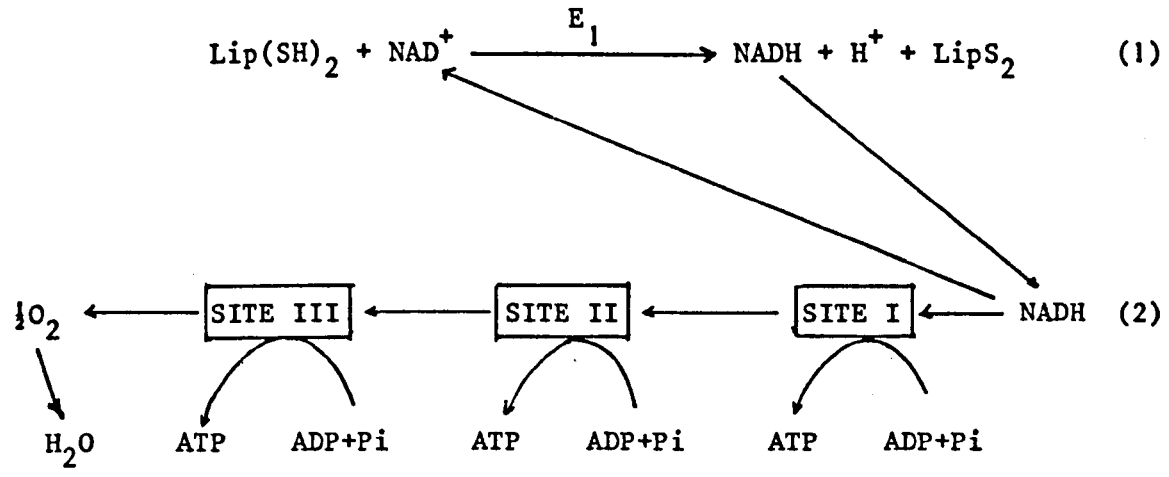
4.6 DISCUSSION

The results presented in this chapter confirm previous findings (132) that dihydrolipoic acid is able to drive ATP synthesis in intact beef heart mitochondria. However, ATP synthesis in submitochondrial particles was dependent on the addition of NAD^+ (Table 4.4). Oleoyl-lipoate and oleoyl-phosphate were found to be inactive as substrates for generating ATP (Tables 4.7-4.8). Like dihydrolipoic acid, oleoyl-lipoate and oleoyl-phosphate were found to be incapable of driving the energy-linked transhydrogenase activity in submitochondrial particles (Table 4.9).

The evidence provided indicates that dihydrolipoic acid does not drive ATP synthesis in mitochondria or submitochondrial

particles by acting as a proton translocator (243) or a high energy chemical intermediate (132), but by acting as a respiratory substrate. This is illustrated by the results presented in Tables 4.3 and 4.4, which show that ATP synthesis in mitochondria and submitochondrial particles is dependent upon the oxidation of dihydrolipoic acid. Inhibition of dihydrolipoic acid oxidation in mitochondria and submitochondrial particles in the presence of NAD^+, by the respiratory inhibitors rotenone and/or antimycin A also causes inhibition of ATP synthesis. Further support for a respiratory substrate role for dihydrolipoic acid is obtained in the work of Griffiths (132) who showed that cyanide inhibition (at site 3) of dihydrolipoic acid oxidation in mitochondria also resulted in inhibition of ATP synthesis. The findings that rotenone, antimycin A (Tables 4.1, 4.3-4.5) or cyanide alone (132) inhibited dihydrolipoic acid oxidation and ATP synthesis in mitochondria, indicate that the electrons deriving from dihydrolipoic acid oxidation are donated to the respiratory chain at site I (see Fig. 4.2). However, the inability of submitochondrial particles to catalyse the oxidation of dihydrolipoic acid in the absence of NAD^+, indicates that the electrons deriving from dihydrolipoic acid are not donated directly to the respiratory chain as suggested by Orlando (251), but are donated indirectly, via, the intermediate electron carrier NAD^+.

The evidence provided, thus suggests that the oxidation of dihydrolipoic acid in mitochondria and the concomitant synthesis of ATP occurs in two steps. The mechanism of the overall process is shown in Fig. 4.2. The first step of the process is the reduction of NAD^+ to NADH by dihydrolipoic acid (Fig. 4.2 reaction 1). This reaction is catalysed by the dihydrolipoic acid reductase (E_1). The second step of the process is the oxidation of the NADH produced in



Overall reaction is

$$Lip(SH)_2 + 3ADP + 3Pi + \tfrac{1}{2}O_2 \rightarrow LipS_2 + H_2O + 3ATP \qquad (3)$$

Fig. 4.2

Mechanism for dihydrolipoic acid driven ATP synthesis in beef heart mitochondria.

the first step, (reaction 1), by the respiratory chain, resulting in the synthesis of ATP in coupled mitochondria. The mechanism proposed in Fig. 4.2 is similar to that proposed for β-hydroxybutyrate driven oxidative phosphorylation in mitochondria (252). The proposed mechanism (Fig. 4.2) is supported by the fact that oxidised lipoic acid (α-lipoic acid) does not drive ATP synthesis (Tables 4.1 and 4.2) and by the observed $Pi/Lip(SH)_2$ (≡Pi/2e or Pi/O) ratio of 2.4 in mitochondria (Table 4.3). A $Pi/Lip(SH)_2$ ratio of 3.0 in mitochondria has been reported previously (132).

The inability of submitochondrial particles to catalyse dihydrolipoic acid oxidation or dihydrolipoic acid driven ATP synthesis in the absence of NAD^+, can therefore be attributed to the loss of the cofactor (NAD^+) during the preparation of submitochondrial particles from whole mitochondria. Similarly, the loss of cofactor and loss of active respiratory chain would also explain the inability of

complex V to catalyse dihydrolipoic acid oxidation, or dihydrolipoic acid driven ATP synthesis (Table 4.8, see ref. 132).

Support for the mechanism outlined in Fig. 4.2 is enhanced by the finding that the dihydrolipoic acid reductase, E_1, proposed in Fig. 4.2, is present in mitochondria and on well-washed submitochondrial particles (see Appendix II). The enzyme activity could not be removed from the submitochondrial particles by washing with 0.1% Triton, suggesting that the enzyme was tightly bound to the membrane. The enzyme was found to co-purify with the F_1 ATPase when the chloroform extraction method of Tyler and Webb (152) was used. However, the extracted enzyme could be separated from F_1 ATPase by ion exchange chromatography on a DEAE-Sephadex column (see Appendix II). The characteristic properties of the reductase such as, K_m, V_m and pH optimum, were found to be independent of the presence of the membrane. Evidence for the presence of a mitochondrial dihydrolipoic acid reductase has been reported by a number of workers (253-254), many of whom have suggested that there were more than one type of dihydrolipoic acid reductase present.

The reduction potential of the pig heart dihydrolipoic acid reductase has been determined by L. J. Reed (206) from the extent of reduction at different $NADH/NAD^+$ and $Lip(SH)_2/LipS_2$ ratios. Values of 0.32 ± 0.01 V and 0.325 V at pH 7.0 and 25° C were obtained respectively. These values are consistent with the reversibility of reaction 1 of Fig. 4.2, since the energy change in going in either direction is virtually zero. The direction of the reaction will, therefore, depend on the relative concentration of NADH, NAD^+, $Lip(SH)_2$ and $LipS_2$ and also the pH of the reaction medium. Investigation of the dependence of the reductase activity on pH, showed that the pH optima of the forward reaction and reverse reaction were pH 8.0

and pH 6.3 respectively (see Appendix II). Thus, under the conditions of the phosphorylation experiment (NAD^+ > NADH, $Lip(SH)_2$>$LipS_2$, and pH 7.4) the forward reaction, that is, the reduction of NAD^+ to NADH, is the favoured reaction. The reaction is further driven forward due to the fact that the NADH produced (reaction 1, Fig. 4.2) is oxidised by the respiratory chain (reaction 2, Fig. 4.2).

The fact that dihydrolipoic acid driven ATP synthesis is inhibited by inhibitors and uncouplers of oxidative phosphorylation (Table 4.5), indicate that a typical energy transfer reaction is occurring, similar to that occurring during succinate driven oxidative phosphorylation. The evidence provided, indicated that dihydrolipoic acid drives ATP synthesis in mitochondria, by acting as a respiratory substrate (cf. β-hydroxybutyrate), and thus refutes the cofactor roles proposed previously by other authors (132, 243).

Oleoyl-lipoate and oleoyl-phosphate were found to be incapable of driving ATP synthesis catalysed by either, mitochondria, submitochondrial particles or complex V. Although catalytic amounts (5-10 nmol) of oleoyl-lipoate or oleoyl-phosphate did not have any effect on succinate driven oxidative phosphorylation in mitochondria, or submitochondrial particles, substrate quantities (1-2 μmol) were found to uncouple succinate driven oxidative phosphorylation (Table 4.6). These results are in agreement with those reported by D. Dickinson (250) and Beauclerk (223) who have shown that oleoyl-lipoate and oleoyl-phosphate does not drive ATP synthesis. The results, however, conflict with the earlier reports of Griffiths (152) and Criddle (256), who have demonstrated oleoyl-lipoate and oleoyl-phosphate driven ATP synthesis. However, it is rather surprising that oleoyl-lipoate and oleoyl-phosphate should have been found to drive ATP synthesis in submitochondrial particles, since substrate concentrations

of these compounds inhibited succinate driven oxidative phosphorylation. It is inconceivable that oleoyl-lipoate and oleoyl-phosphate should uncouple succinate driven oxidative phsophorylation, but still drive an uncoupler sensitive ATP synthesis. Furthermore, oleic acid, one of the final products of the proposed reaction (Fig. 5.1) for the oleoyl-lipoate driven ATP synthesis and oleoyl-phosphate are potent uncouplers of oxidative phosphorylation (see Chapter 5).

The inability of dihydrolipoic acid, oleoyl-lipoate or oleoyl-phosphate to drives the energy-linked NADH/NADP transhydrogenase reaction of submitochondrial particles, indicates that these compounds are not high energy chemical intermediates, nor do they give rise directly to an energised state. The fact that oleoyl-lipoate and oleoyl-phosphate inhibits ATP driven transhydrogenase reaction, but does not significantly affect the succinate driven reaction (Table 4.9) indicates that oleoyl-lipoate acts directly upon the ATP-synthase/ATPase energy coupling complex.

The results presented in this Chapter demonstrates that dihydrolipoic acid drive ATP synthesis in mitochondria by acting as a NAD-linked respiratory substrate, in a similar way to β-hydroxy butyrate. The results do not provide any support for either the chemosmotic or chemical coupling hypothesis of oxidative phosphorylation, since dihydrolipoic acid does not act as a proton translocator or high energy chemical intermediate.

Although dihydrolipoic acid has been shown to drive ATP synthesis in mitochondria, [Table 4.1 (132)] and to stimulate photophosphorylation in chloroplast (256) and NADH/NADP transhydrogenase activity in Rhodopseudomonas spheroides (251); these reactions might be biologically insignificant and artifactual, since in these reactions it acts as an artificial electron donor. At present there

is no convincing evidence of any other natural function of lipoic acid other than its established role in CoA and NAD-linked oxidative decarboxylation of α-keto acids (245).

CHAPTER 5

5. THE EFFECT OF FATTY ACIDS AND THEIR ACYL-ESTERS ON OS-ATPase, OXIDATIVE PHOSPHORYLATION AND ENERGY-LINKED REACTIONS OF MITOCHONDRIA

5.1 INTRODUCTION

Long chain fatty acids occupy an important position in the energy metabolism of the cell. They are good respiratory substrates whose oxidation is coupled to the generation of ATP (183), but they can also be potent uncouplers of oxidative phosphorylation (184-186). It has therefore been suggested that fatty acids (or their acyl-esters) may play a role in the regulation of the degree of coupling between (184-189) respiration and the synthesis of ATP. Two different mechanisms have been proposed to explain the regulation of the degree of coupling between mitochondrial respiration and phosphorylation by fatty acid. Joel et al. (190), Prusiner et al. (191) and a number of other workers (192-194) have proposed that fatty acids regulate oxidative phosphorylation by acting as uncouplers of oxidative phosphorylation (cf. FCCP Appendix I). Such a regulatory role of fatty acid has recently been postulated for the brown adipose tissue for which the main physiological role is thermoneogenesis (192-194). In brown adipose tissue, the fatty acids released by lipolysis are absorbed onto the mitochondria causing uncoupling of oxidative phosphorylation, while ensuring maximal rate of respiration. The energy released by oxidation is transformed directly to heat. Evidence in support of an uncoupler mode of action of fatty acids, have been provided by Falcone and Mao (195) and a number of other workers (193-196) who have shown that fatty acids uncouple oxidative phosphorylation and ^{32}P-ATP exchange in mitochondria. These workers (193-196) also showed that the inhibitory action of fatty acids could be reversed by addition of bovine serum albumin.

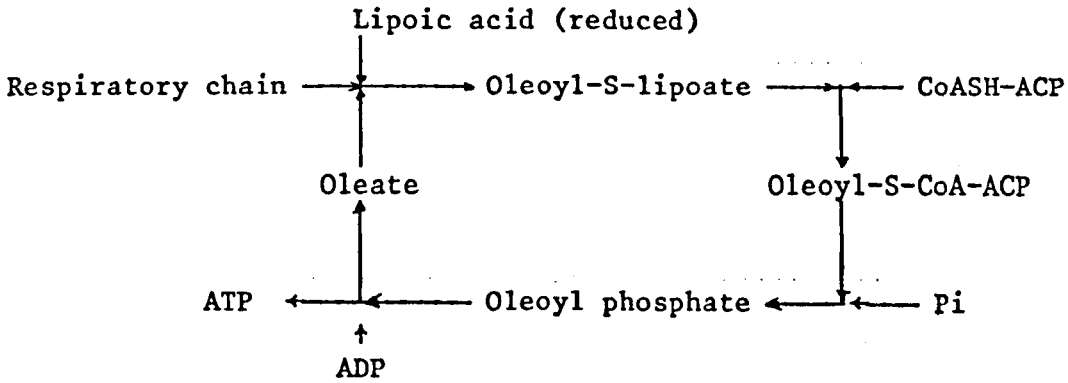
Recently, however, fatty acids have been proposed to play a specific cofactor role in the terminal reactions of mitochondrial oxidative phosphorylation. The possibility that fatty acids and their acyl esters might act as intermediates of oxidative phosphorylation, was first proposed by Bode and Klingenberg (197), who suggested that the energy required for the formation of the acyl-S-CoA esters of fatty acids could be supplied directly by the high-energy intermediates of oxidative phosphorylation. Evidence that this process occurred in rat liver mitochondria was brought forward by Wojtczak et al. (198-199). However, Pierrot et al. (200) have demonstrated that the DNP and oligomycin insensitive fatty acid activation observed by Bode and Klingenber (197) and Wojtczak et al. (198, 199) was due to substrate-level phosphorylation linked to the oxidation of the oxo-glutarate step of the TCA cycle. Inhibition of substrate-level phosphorylation with arsenite completely abolishes fatty acid activation in DNP treated mitochondria. The possibility that high-energy intermediates of oxidative phosphorylation could activate fatty acids without the intermediate formation of nucleotide triphosphate must, therefore, be ruled out.

More recently, however, a cofactor role for lipoic acid and unsaturated fatty acids in oxidative phosphorylation has been postulated, and net synthesis of ATP catalysed by mitochondrial particles and ATP synthase preparations has been demonstrated utilising oleoyl-S-lipoate and oleoyl phosphate as substrates (132). Oleoyl phosphate dependent ATP synthesis by submitochondrial particles and ATP synthase preparations (complex V) was shown to be insensitive to uncouplers such as FCCP, TTFB and '1799' and to oligomycin and triethyltin, but was sensitive to DCCD and F_1-ATPase inhibitors such as efrapeptin (132). These findings led Griffiths (132) to propose

the 'oleoyl cycle' (Fig. 5.1) as a 'framework' for the investigation of the roles played by fatty acid, CoA and acyl-S-CoA esters in oxidative phosphorylation. In the 'oleoyl cycle' hypothesis, Griffiths proposed that the terminal steps of oxidative phosphorylation proceeded by a series of reactions involving acylation, transacylation phosphorylation and transphorylation (Fig. 5.1). Available evidence in support of such a series of reactions is rather limited, and are contrary to those reported by a number of workers (191-196). Because of the controversial nature of the proposed 'oleoyl-cycle' of oxidation (and controversy always surrounds any new proposal for oxidative phosphorylation) we decided to investigate a possible cofactor role for fatty acids (especially oleic acid) and their derivatives in oxidative phosphorylation.

In this Chapter we look at the effect of fatty acids and their acyl ester derivatives on oxidative phosphorylation, ATPase activity and other energy-linked reactions of mitochondria.

Fig. 5.1 The oleoyl cycle of oxidative phosphorylation (224)



ACP ≡ acyl carrier protein

5.2 MATERIALS

All reagents were of AnalaR grade. All fatty acids, and their acyl esters were obtained from Sigma Chemical Company. Mitochondria and submitochondrial particles were prepared as described in the Methods section of Chapter 2. Oleoyl-phosphate palmitoyl-phosphate and elaidoyl-phosphate were prepared as described in the Methods section of Chapter 4. ^{32}Pi was obtained from the Radiochemical Centre, Amersham.

5.3 METHODS

Oxidative phosphorylation, ATPase activity, and trans-hydrogenase activity were measured as described in the Methods section of Chapter 2.

ATP-Pi exchange

ATP-Pi exchange activity was determined as described by Stigall et al. (241). To 0.5 ml of a solution containing 50 mM tris-Cl, pH 7.5; 500 mM sucrose; 30 mM $MgSO_4$; 40 mM potassium phosphate (pH 7.5), was added 0.5 x 10^6 c.p.m. of carrier free ^{32}Pi and water to bring the volume to 0.90-0.95 ml. Submitochondrial particles, 1 mg (in 50-100 µl volume)were then added and the mixture preincubated with any inhibitors or effectors for 5 minutes at 30° C. The reaction was started by adding 12 µmoles ATP (50 µl 0.24 M ATP, pH 7.5) and terminated after 10 minutes by addition of 0.5 ml 1 M PCA (perchloric acid) or 0.5 ml 10% trichloroacetic acid (TCA). Coagulated protein was removed by bench centrifugation and a 0.5 ml aliquot from the supernatant taken for determination of ^{32}P labelled ATP by a method based on that described by Pullman (242).

The 0.5 ml assay aliquot was taken into a 10 ml glass test tube containing 1.0 ml 5%ammonium molybdate in 5 N H_2SO_4; 1 ml acetone was then added and the mixture mixed vigorously on a vortex shaker. 0.1 ml bromine water was added followed by 5 mls water-saturated isobutanol/toluene (1:1, v/v). The mixture was vortex mixed for 30 seconds and the upper layer removed by aspiration. The process was repeated. 5 ml of water-saturated isobutanol was then added, the mixture vortexed and the upper layer removed by aspiration. The remaining aqueous layer was extracted twice by water-saturated diethylether (5 ml) which was also removed by aspiration. Aliquots from the remaining aqueous layer was taken for determination of ^{32}P by liquid scintillation counting.

5.4 RESULTS AND DISCUSSION

Fig. 5.2 and Fig. 5.3 show the effect of various fatty acids on succinate driven oxidative phosphorylation in beef heart mitochondria and submitochondrial particles respectively. It can be seen that all the fatty acids investigated inhibit oxidative phosphorylation in mitochondria and submitochondrial particles and that the effectiveness of a fatty acid as an inhibitor depended on the nature of the fatty acid. For example, oleic acid, petroselenic acid, palmitic acid, stearic acid, elaidic acid and cis-linoleic acid at 80-100 nmol/mg protein fully inhibit oxidative phosphorylation (Table 5.1) in mitochondria. Oleic acid, elaidic acid and petroselenic acid were found to be twice as potent as the other fatty acids, causing 80-90% inhibition of ATP synthesis at 50 nmol/mg protein (Fig. 5.2, Table 5.1). Most of the fatty acids investigated were found to be much less effective in inhibiting oxidative phosphorylation in submitochondrial particles than in mitochondria

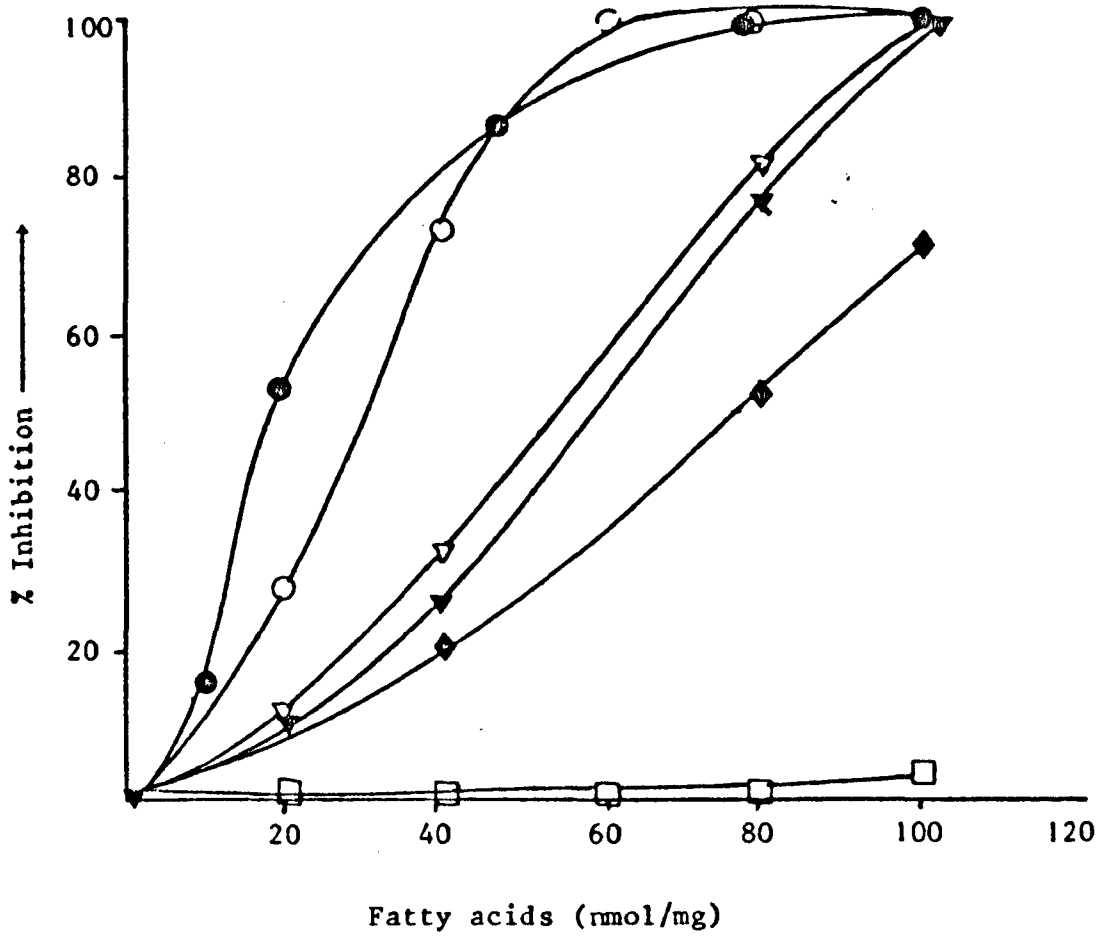


Fig. 5.2 Inhibition of succinate driven oxidative phosphorylation in beef heart mitochondria by fatty acids

Oxidative phosphorylation was assayed as described in Materials and Methods. Mitochondria (1 mg) were preincubated for 5 minutes at 30° C in phosphorylation medium containing 2 µg rotenone and varying concentrations of fatty acid/derivatives. ● , oleic acid; ○ , petroselenic acid; ▽ , stearic acid; ▼ , palmitic acid; ◆ , cis-vaselenic; □ , methyl palmitate. The reaction was initiated by addition of 20 µmol succinate and allowed to run for 20 minutes. The specific rate of ATP synthesis of mitochondria in a system containing no fatty acid is 135 nmol ATP synthesised/min/mg protein. Experimental data has been presented in the figure as a per cent inhibition of this original value. Each point is the average of 4 duplicates.

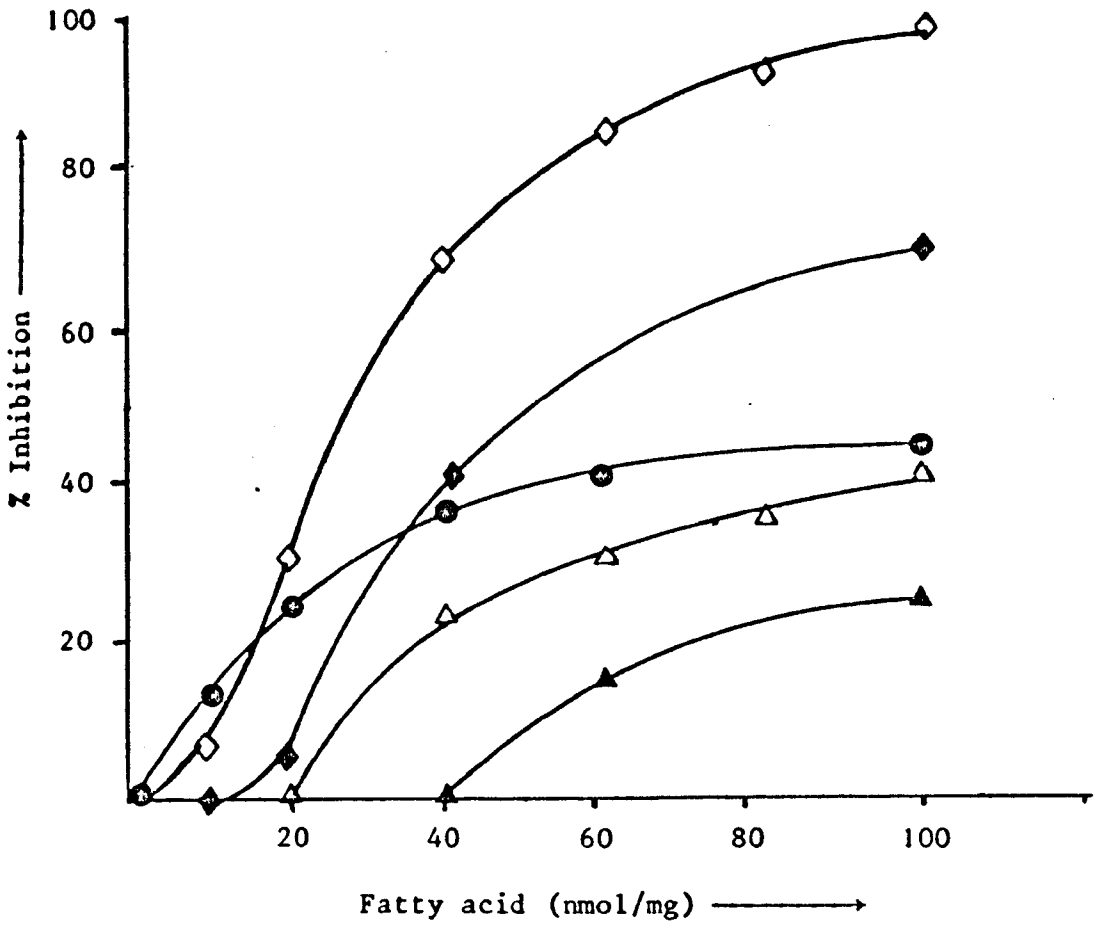


Fig. 5.3 Fatty acid/derivatives inhibition of succinate driven oxidative phosphorylation in submitochondrial particles

All conditions for the measurement of ATP synthesis were as described in the legend of Fig. 6.2, except that submitochondrial particles and not mitochondria were used in the assay. ◇ , oleic acid; ◆ , petroselenic acid; ● , stearic acid; △ , palmitic acid. Specific rate of ATP synthesis in controlled samples containing no fatty acid is 110 nmol ATP synthesised/min/mg protein. Experimental data has been presented in the figure as a per cent inhibition of this original value. Each data point is the average of duplicates.

Table 5.1 Effect of fatty acids (derivatives) on succinate driven oxidative phosphorylation in mitochondria

Fatty acid/derivative		ATP synthesis (nmol/min/mg)	% Activity
None		135.0	100.0
Oleic acid	(100 nmol)	0.0	0.0
Methyl oleate	(100 nmol)	117.5	87.0
Ethyl oleate	(100 nmol)	126.5	93.5
Butyl oleate	(100 nmol)	135.0	100.0
Oleoyl phosphate	(100 nmol)	0.0	0.0
Oleoyl-SCoA	(25 nmol)	15.0	11.1
Palmitic acid	(100 nmol)	0.0	0.0
Methyl palmitate	(100 nmol)	135.0	100.0
Ethyl palmitate	(100 nmol)	125.0	92.0
Palmitoyl phosphate	(100 nmol)	0.0	0.0
Palmitoyl-SCoA	(30 nmol)	37.5	27.5
Elaidic acid	(100 nmol)	0.0	0.0
Elaidoyl phosphate	(100 nmol)	0.0	0.0
Petroselenic acid	(100 nmol)	0.0	0.0
FCCP	(5 µg)	0.0	0.0

ATP synthesis was estimated in a glucose-hexokinase trap system as described in Materials and Methods. Mitochondria were suspended in 0.25 M sucrose, 10 mM tris-HCl pH 7.4 at 20 mg/ml. Aliquots (1 mg protein) were added to the phosphorylation medium containing 2 µg rotenone and the fatty acids/derivatives presented in the Table. Total volume 1.0 ml. The amount of each fatty acid/derivatives added is presented in brackets. The mitochondria were preincubated for 5 minutes with inhibitors and fatty acid/derivatives, prior to initiation of the assay by addition of 20 µmol succinate. The data presented in the table represents the average of 4 duplicates.

(Figs. 5.2, 5.3) producing on average 30-40% inhibition in submitochondrial particles compared with 70-100% inhibition in mitochondria at 100 nmol/mg protein. However, oleic acid, petroselenic and elaidic acid were found to be just as effective as inhibitors of oxidative phosphorylation in submitochondrial particles as in mitochondria, causing 80-100% inhibition (Tables 5.1 and 5.2). The results presented in Tables 5.1 and 5.2 show that the methyl, ethyl and butyl esters of oleic acid and palmitic acid had no effect on succinate ATP synthesis in mitochondria or submitochondrial particles. However, the acyl-phosphates of oleic, palmitic and elaidic acid were found to inhibit succinate driven oxidative phosphorylation in mitochondria and submitochondrial particles, while the acyl-S-CoA derivatives only inhibited ATP synthesis in intact mitochondria. 10-15 nmol/mg protein of acyl-S-CoA was found to inhibit ATP-synthesis in intact mitochondria by 70-80%.

Except for oleic acid and oleoyl-phosphate all fatty acids and their acyl-ester derivatives were found to have no significant effect on ATP-driven transhydrogenase activity of beef heart submitochondrial particles, not even at concentrations exceeding 500 nmol/mg protein (Table 5.3, Fig. 5.4). Oleic acid and oleoyl-phosphate on the other hand were found to inhibit ATP-driven transhydrogenase by 70% and 65% respectively at 500 nmol/mg protein. However, like the other fatty acids and their acyl ester derivatives, oleic acid and oleoyl-phosphate were found to have no effect on the succinate driven transhydrogenase activity (Table 5.3).

The results presented in Table 5.4 show that the Pi-ATP exchange activity of submitochondrial particles was significantly inhibited by oleic acid, elaidic acid and oleoyl-phosphate at 100 nmol/mg protein. However, palmitic acid, its acyl ester

Table 5.2 Effect of fatty acids/derivatives on succinate driven oxidative phosphorylation in submitochondrial particles

Fatty acid/derivative		ATP synthesis (nmol/min/mg)	% Activity
None		110.0	100.0
Oleic acid	(100 nmol)	0.0	
Methyl oleate	(100 nmol)	110.0	100.0
Ethyl oleate	(100 nmol)	110.0	100.0
Butyl oleate	(100 nmol)	110.0	100.0
Oleoyl phosphate	(100 nmol)	0.0	0.0
Oleoyl-SCoA	(40 nmol)	110.0	100.0
Palmitic acid	(100 nmol)	65.0	57.6
Methyl palmitate	(100 nmol)	110.0	100
Palmitoyl-SCoA	(50 nmol)	92.0	84.0
Elaidic acid	(100 nmol)	31.0	28.0
Elaidoyl phosphate	(100 nmol)	0.0	0.0
Carboxyatractyloside	(1 µg)	106.0	96.2
Petroselenic acid (100 nmol)		24.0	23.0

Oxidative phosphorylation was assayed as described in Methods. Submitochondrial particles (1 mg) were preincubated in 1 ml phosphorylation buffer containing 2 µg rotenone and fatty acid/derivatives (amount added indicated in brackets) for 5 minutes. The reaction was initiated by addition of 20 µmol succinate and allowed to run for 20 minutes. The data presented in the Table represents the average of 4 duplicates.

derivatives, oleoyl-S-CoA and oleoyl-S-lipoates did not significantly affect the Pi-ATP exchange reaction (Table 5.4). These results are in complete contrast with those previously reported by Hyams et al., who found that oleic acid and its acyl ester derivatives were able to stimulate Pi-ATP exchange activity more than 20 fold (225).

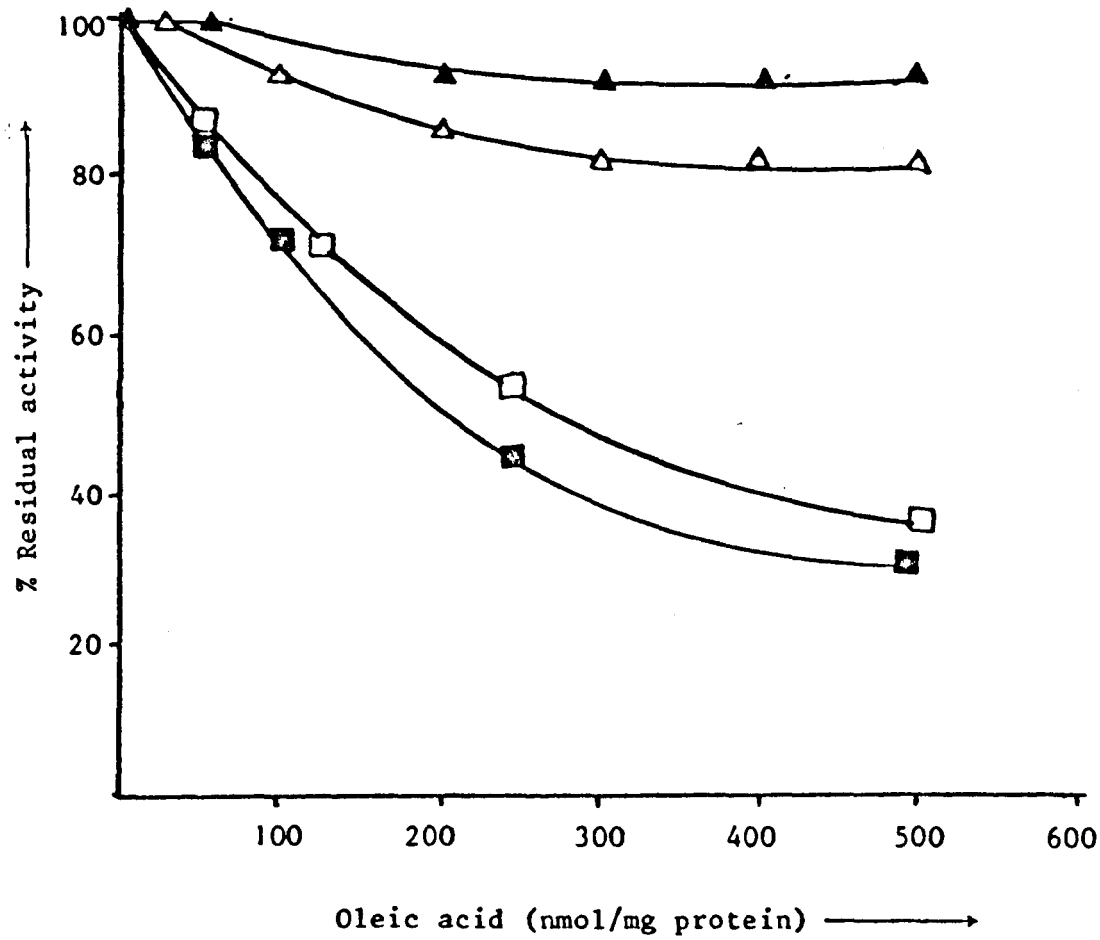


Fig. 5.4 Effects of fatty acids/derivatives on ATP-driven transhydrogenase

Conditions of ATP-driven transhydrogenase assay were as described in Table 5.3. Submitochondrial particles were incubated at various concentrations of fatty acids/derivatives for 20 minutes at 25° C. 1 mg aliquots were then assayed for ATP driven transhydrogenase. The specific activity of the original submitochondrial particles is 36.3 ± 0.8 nmol NADPH formed/min/mg protein. Experimental data are expressed in the figure as a per cent of this original value. Each data point is the average of duplicates. (▲), methyl palmitate; (△), palmitic acid; (■), oleic acid and (□) oleoyl phosphate.

Table 5.3 Effect of fatty acids/derivatives on ATP-driven and succinate driven transhydrogenase activity of submitochondrial particles

Fatty acid/derivatives		ATP-driven nmol $NADPH_f$/ min/mg	Succinate-driven nmol $NADPH_f$/ min/mg
None		36.3	60.0
Oleic acid	(200 nmol)	18.0	60.0
Methyl oleate	(200 nmol)	35.8	60.0
Ethyl oleate	(200 nmol)	36.0	60.0
Butyl oleate	(200 nmol)	36.7	60.0
Oleoyl phosphate	(200 nmol)	25.0	58.0
Oleoyl CoA	(50 nmol)	36.4	60.0
Palmitic acid	(200 nmol)	30.2	60.0
Methyl palmitate	(200 nmol)	36.0	60.0
Palmitoyl phosphate	(200 nmol)	36.4	60.0
Palmitoyl-SCoA	(70 nmol)	36.3	60.0
Elaidic acid	(200 nmol)	36.5	60.0
Elaidoyl phosphate	(200 nmol)	35.9	60.0

Transhydrogenase activity was assayed as described in Materials and Methods. Submitochondrial particles (1 mg) were preincubated in transhydrogenase assay medium containing 1 µg rotenone (± 1 µg antimycin A), 50 mM tris-Cl pH 8.0, 0.25 M sucrose, 1 mM EDTA, 66 µM NAD^+, 0.33 mM $NADP^+$, 10 mM ethanol, 10 units alcohol dehydrogenase, and varying concentrations of various fatty acids/derivatives for 5 minutes. The reaction was then initiated by adding ATP (12 µmol) or succinate (50 µmol). $NADPH_f$ = NADPH formed. The data presented represents the average of duplicates.

The inhibition of oxidative phosphorylation by fatty acids has been attributed to several different modes of action (198, 200). They have been proposed to act as uncouplers, having a similar mode of action to that of 2,4-dinitrophenol (198, 200). Fatty acids are also powerful swelling agents of mitochondria

Table 5.4 The effect of fatty acids/derivatives on ^{32}P-ATP exchange catalysed by submitochondrial particles

		Pi-ATP Exchange activity nmoles/min/mg	% Activity
None		280 ± 20	100.0
Palmitic acid	(100 nmol)	260 ± 10	93.0
Palmitoyl phosphate	(100 nmol)	260 ± 12	93.0
Palmitoyl-SCoA	(50 nmol)	280 ± 10	100.0
Elaidic acid	(100 nmol)	160 ± 25	57.0
Oleic acid	(100 nmol)	95 ± 15	34.0
Oleoyl phosphate	(100 nmol)	100 ± 12	35.7
Oleoyl-SCoA	(40 nmol)	260 ± 18	93.0
Oleoyl-S-lipoate	(100 nmol)	210 ± 17	75.0
Oligomycin	(2 µg)	0	0.0
DNP	(2 µg)	0	0.0
TTFB	(2 µg)	0	0.0

Pi-ATP exchange activity was assayed as described in Materials and Methods. The reaction mixture 25 mM tris-SO_4, pH 7.5, 15 mM $MgCl_2$, 20 mM potassium phosphate, 250 mM sucrose 0.5×10^6 c.p.m. ^{32}P and 1.0 mg submitochondrial particles. The reaction mixture was preincubated with added fatty acid/derivatives ± inhibitors (2 µg) (added as methanolic solutions) for 5 minutes at 30° C. The reaction was initiated by addition of 50 µl. 0.24 M ATP pH 7.5. Final volume 1.0 ml. Each value presented is the mean ± s.d. of five duplicates. No correction for ATP hydrolysis was made in calculation of Pi-ATP exchange activity. The amount of fatty acid/derivatives added is presented in brackets.

(186, 226) and their uncoupling action has been correlated with their swelling action. Both effects have been shown by Wojtczak and others (186, 198, 198) to be strongly dependent on the length of the carbon chain and on the presence or absence of double bonds, the most potent being the longchain unsaturated fatty acids. An uncoupling

mode of action for fatty acids on oxidative phosphorylation is supported by the findings that, fatty acid at a concentration (100 nmol/mg) which maximally stimulate succinate oxidation (186, 198), inhibits ^{32}Pi-ATP exchange (Table 5.4 and ref. 198) and ATP synthesis (Figs. 5.2 and 5.3) does not induce significant mitochondrial swelling (186, 189, 198) or affect the OS-ATPase activity. Fatty acids are thus proposed to uncouple oxidative phosphorylation and other energy-linked reactions by acting as a protonophore which transports proton across the mitochondrial membrane, resulting in the elimination of the proton gradient. Recent studies by Carafoli and Rossi (227) has shown that fatty acids facilitate the transport of protons across the mitochondrial membrane. The uncoupling action of fatty acids, thus depend on the ability of the fatty acid to transport protons, which in turn will depend on the presence of a 'free' carboxylic group and on its pKa. This is borne out by the fact that the methyl, ethyl and butyl esters of oleic acid and palmitic acid does not affect oxidative phosphorylation or energy-linked reactions (Tables 5.1-5.3). The acyl-phosphates, however, because of their anionic nature, are potent uncouplers of oxidative phosphorylation and energy-linked reactions (Table 5.1-5.4).

The results presented in Tables 5.1 and 5.2, also show that although the CoA esters of oleic and palmitic acid do not affect ATP synthesis in submitochondrial particles at low concentration (30 nmol/mg protein), they inhibit ATP synthesis in intact mitochondria by 70-80% at 20-30 nmol/mg protein. The inhibition of ATP synthesis by these acyl-CoA esters have been attributed to their inhibition of adenine nucleotide translocation in mitochondria (228-232). This makes the availability of ADP the rate limiting step in oxidative phosphorylation. The inhibition of ADP translocation is supported by the fact that the acyl-CoA esters do not affect succinate driven oxidative phosphorylation in submitochondrial

particles. Acyl-CoA esters have also been shown to bind specifically to the adenine nucleotide translocase (a 30,000 molecular weight protein identical to the one bound by carboxy atractyloside and bongkrekic acid) thus causing inhibition, by blocking a conformational change that is obligatory for the action of the translocase (198, 232). However, in contrast to atractyloside and bongkrekic acid, long chain fatty acyl-CoA esters bind and inhibit the ADP/ATP translocase from both the cytosolic and the matrix sides of the inner membrane (228-232).

A controversial issue which is recognised as one of the most fundamental in bioenergetics is whether or not the ADP/ATP carrier is rate limiting in the overall reaction of oxidative phosphorylation (233). Another important question under consideration is whether or not long chain acyl-CoA esters are physiological effectors of adenine nucleotide translocation (228, 229). The two issues are closely inter-related, and it would seem logical to expect that a reaction that is carefully regulated, is itself rate limiting or vice versa. Davis and Lumeng (234) reported marked effects of palmitoyl CoA on the steady state internal and external phosphorylation potentials generated by respiring mitochondria under experimental conditions where the ADP/ATP carrier was rate limiting. The present results, on the effect of oleoyl and palmitoyl CoA esters on oxidative phosphorylation in beef heart mitochondria presented in Table 5.1, provide independent support for the more recent reports (235-236), which conclude that the adenine nucleotide translocase is the rate limiting step in oxidative phosphorylation.

Peculiar, and not typical of other uncouplers of oxidative phosphorylation, is the effect of fatty acids on ATPase activity in mitochondria (198, 237, 238), submitochondrial particles

and the soluble F_1-ATPase. This is shown on Figs. 5.5-5.9. Under the experimental conditions employed, increasing the amount of oleic acid was found to stimulate the latent ATPase activity in mitochondria (Fig. 5.5), submitochondrial particles (Figs. 5.5, 5.6) and F_1-ATPase (Fig. 5.7), attaining a maximum usually at 400-500 nmol/mg protein. At oleate concentration above 400 nmol/mg protein the ATPase activity of mitochondria and submitochondrial particles becomes insensitive to inhibition by oligomycin, dicyclohexylcarbodiimide (DCCD) and dibutylchloromethyltin chloride (DBCT) (Fig. 5.8). Stimulation of the latent ATPase activity of submitochondrial particles was also obtained with most of the fatty acids studied (Fig. 5.6) but the degree of stimulation were much smaller than that obtained with oleic acid. The stimulation and the sensitivity to oligomycin DBCT, and DCCD can be reversed by serum albumin (Table 5.5 and Fig. 5.9). The results presented on Fig. 5.9 also show that the amount of serum albumin required to completely reverse DBCT inhibition of oleic acid treated submitochondrial particles was dependent upon the amount of oleic acid used, and that the serum albumin did not affect the ATPase activity in submitochondrial particles.

The effects of fatty acids on the ATPase activity of mitochondria, submitochondrial particles and F_1 are contrary to those of 2,4-dinitrophenol and other typical uncouplers, which has been shown to stimulate only the membrane bound oligomycin sensitive ATPase which is irreversible by serum albumin. The effects however are strikingly similar to the action of the surface active agent deoxycholate on mitochondrial ATPase (198). The stimulation of the ATPase activity in intact mitochondria has been suggested (198) to be due to the increased permeability of the mitochondria, brought about through breakdown of the mitochondrial membrane structure

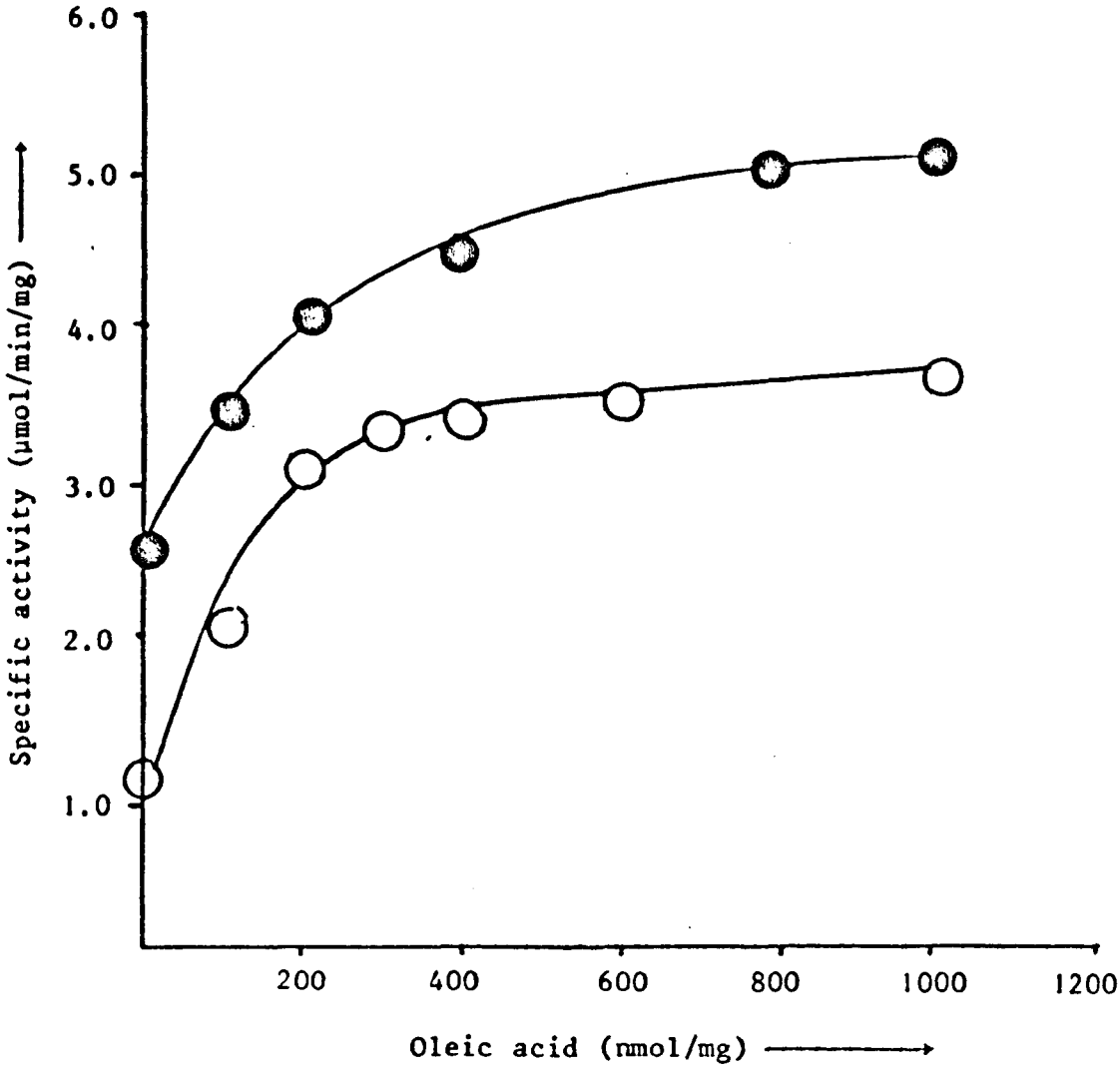


Fig. 5.5 Stimulation of ATPase activity in mitochondria and submitochondrial particles by oleic acid

ATPase activity was assayed as described in Methods. Mitochondria and submitochondrial particles suspended at 10 mg/ml, were preincubated for 30 minutes at 25° C with various concentrations of oleic acid. 10 µl aliquots (100 µg) were then assayed for ATPase activity. The specific activity of the original mitochondria (O) is 1.2 µmol/min/mg protein, and submitochondrial particles (●) is 2.5 µmol/min/mg. Each data point is the average of duplicates.

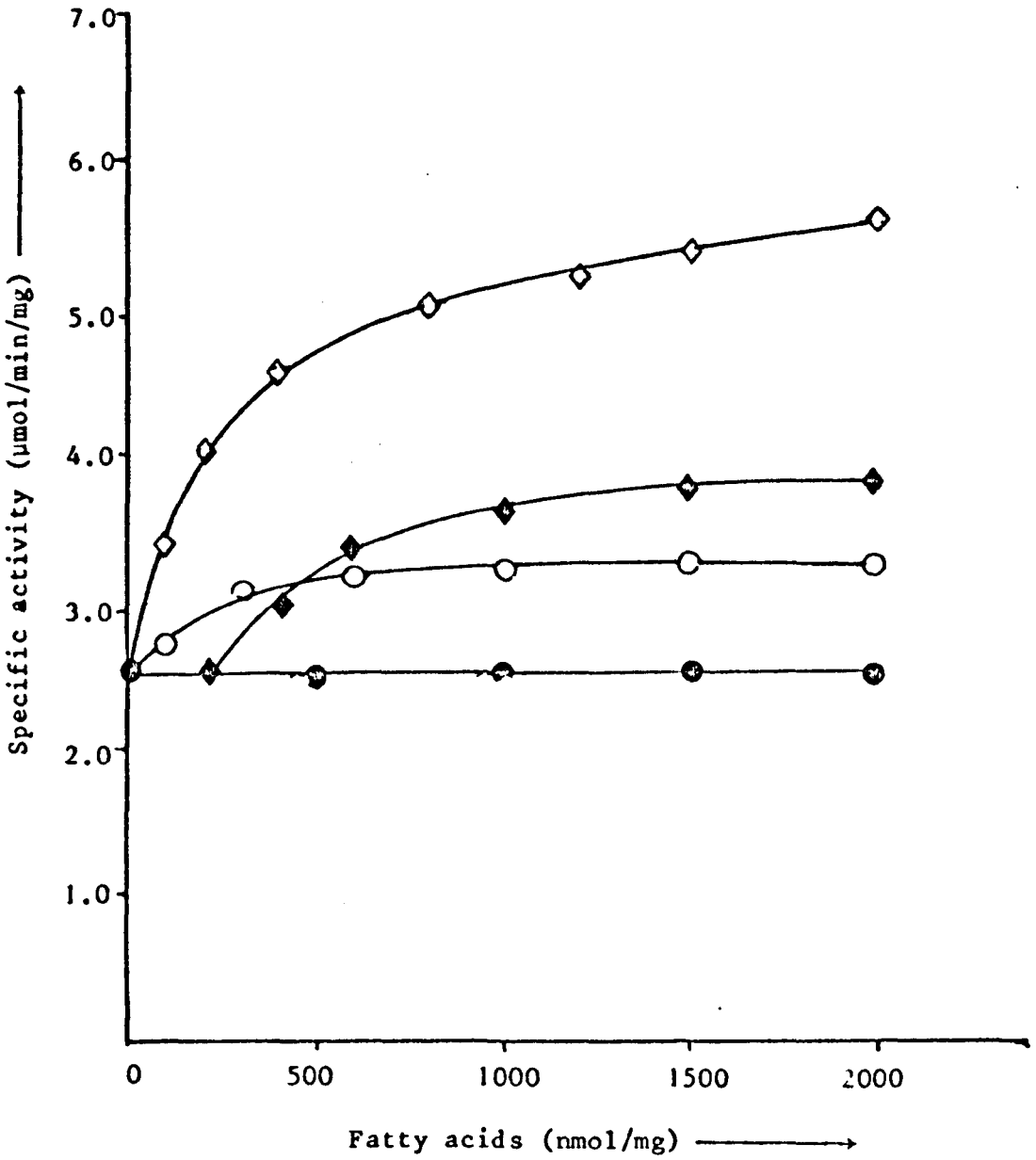


Fig. 5.6 Stimulation of ATPase activity in beef heart submitochondrial particles by fatty acids

Submitochondrial particles were treated with various fatty acid concentrations and assayed for ATPase activity as described in the legend of Fig. 5.5 (◇), oleic acid; (◆), cis-vaselenic acid; (○), elaidic acid; (●), palmitic acid.

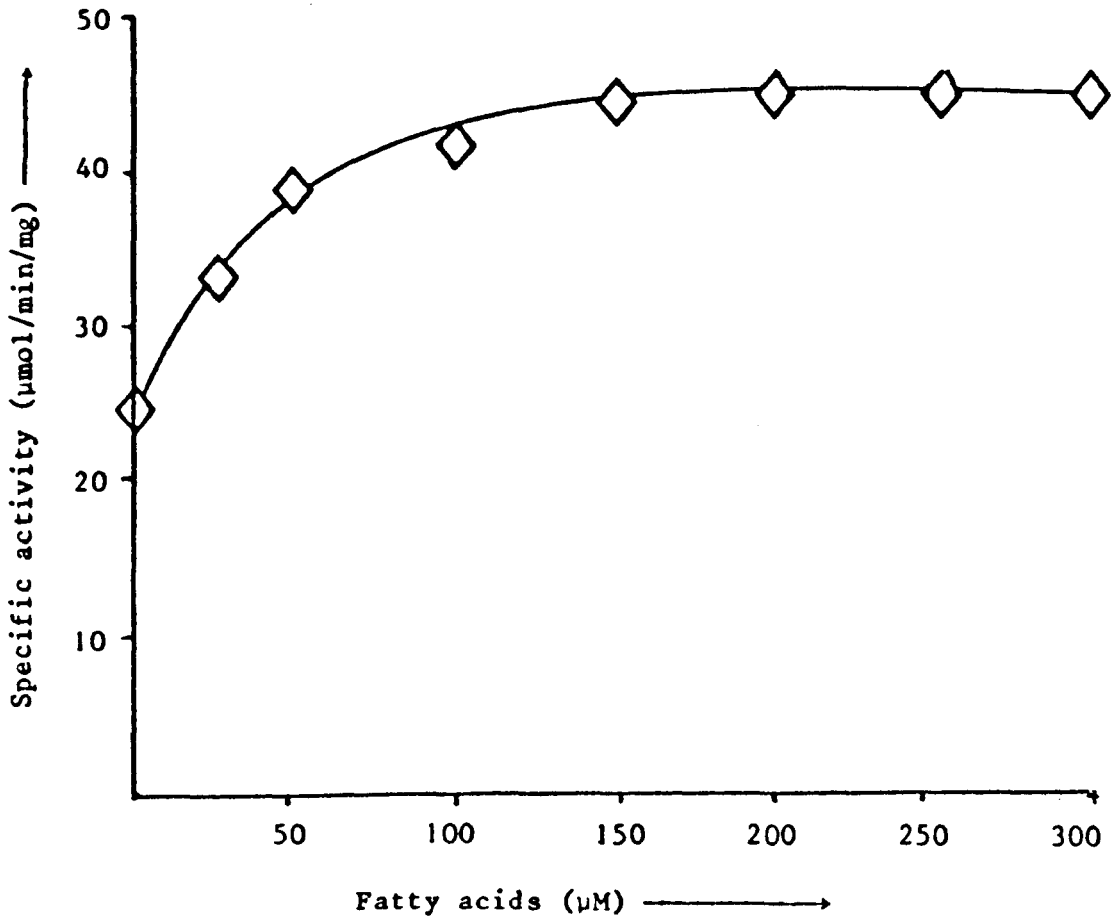


Fig. 5.7 Stimulation of the F_1 ATPase activity by oleic acid

ATPase activity was assayed as described in Methods. F_1 ATPase (10 μg) was preincubated for 5 minutes at 30° C in 1 ml (50 mM tris-Cl. pH 8.5; 5 mM $MgCl_2$) buffer with various concentrations of oleic acid. Each point on the graph represents the mean of five duplicates.

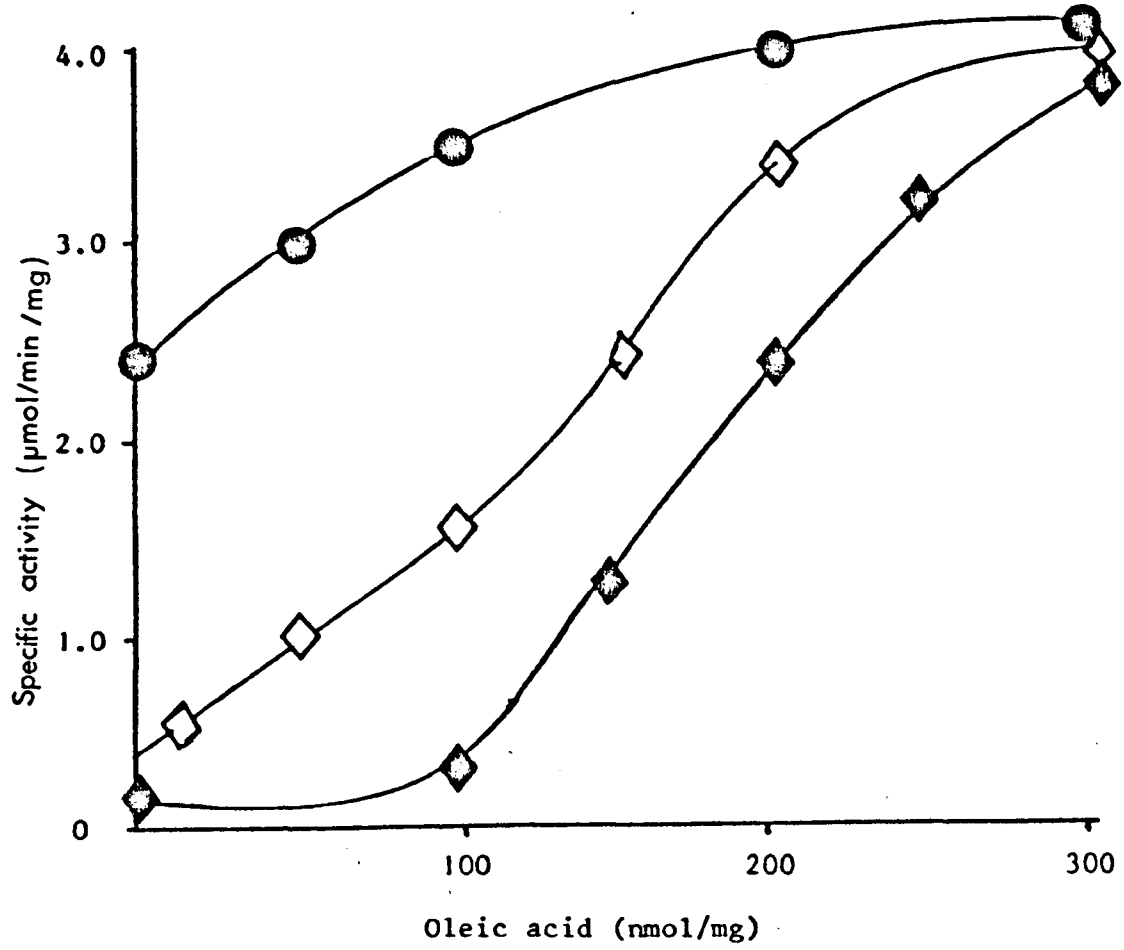


Fig. 5.8 Reversal of DBCT and oligomycin inhibited ATPase activity of submitochondrial particles by oleic acid

ATPase activity was assayed as described in Materials and Methods. Submitochondrial particles suspended at 10 mg/ml pretreated with oligomycin or DBCT (5 nmol/mg) were incubated with various concentration of oleic acid. 10 µl aliquots (100 µg) were removed and assayed for ATPase activity. (●) uninhibited, (◊) inhibited with DBCT, (◆) inhibited with oligomycin.

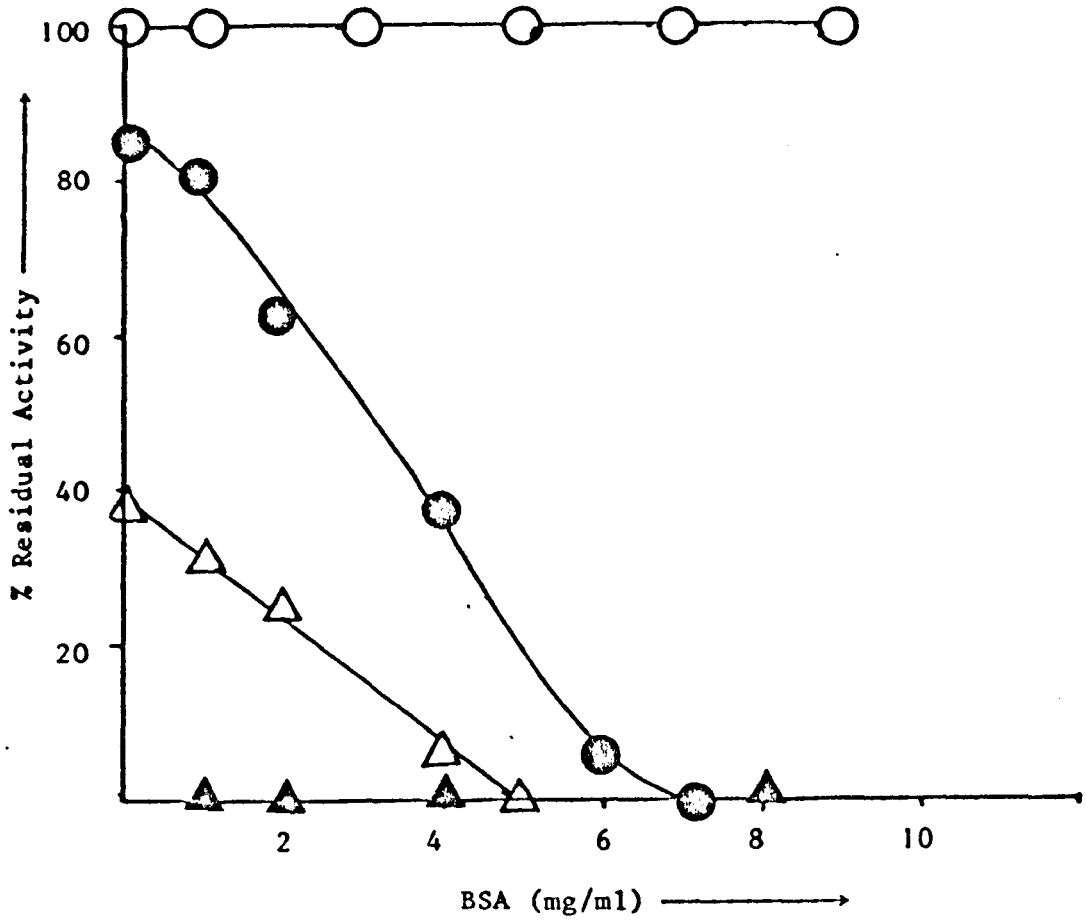


Fig. 5.9 Effect of bovine serum albumin on oleic acid reversal of DBCT inhibited ATPase activity in submitochondrial particles

Submitochondrial particles suspended at 10 mg/ml, pretreated with DBCT (5 nmol/mg protein) were treated with oleic acid at 50 nmol/mg protein (Δ) and 100 nmol/mg protein (●). 100 µg aliquots were then preincubated in 1.0 ml of ATPase buffer containing various concentrations of bovine serum albumin (BSA) for 5 minutes and assayed for ATPase activity. Specific ATPase activity of untreated submitochondrial particles is 2.0 µmol/min/mg protein. Experimental data are presented in the figure as a per cent of this value. (○), not treated with DBCT; (▲) + DBCT - oleic acid.

by oleic acid. Under these conditions the penetration of ATP into the mitochondria is no longer dependent on the rate limiting action of the ADP/ATP translocase. The insensitivity of the ATPase activity under these conditions to atractyloside has been used (198) as evidence to support this explanation. However, although oleate-induced increased permeability might be partially responsible for the increased ATPase activity in mitochondria, increased permeability cannot be used to explain the stimulation of the ATPase activity in submitochondrial particles and F_1-ATPase since the availability of ATP is not a rate limiting factor, nor the inhibitor insensitivity induced by oleate (Fig. 5.7). The simplest explanation is that oleate, due to its detergent effect at high concentration, causes solubilisation of the more active F_1-ATPase from the membrane. The F_1-ATPase activity which is insensitive to oligomycin, DCCD and DBCT being further stimulated by the oleic acid. However, the fact that oleate stimulation of ATPase activity and its induced inhibitor insensitivity can be reversed with serum albumin (Table 5.5), suggests that the F_1-ATPase is not solubilised off the membrane. This was confirmed by using a procedure in which oleate treated submitochondrial particles (500 nmol/mg protein) were centrifuged at 100,000 g for one hour. Using this procedure all the ATPase activity was found to be present in the 'pelleted' membrane fraction.

The results are more suitably explained by the induction of a morphological change in membrane structure, which cause a 'dislocation' of the F_1-ATPase on (not dis-association from) the membrane. This results in increased ATPase activity, and induce inhibitor insensitivity. Removal of the oleate with serum albumin releases the dislocation concommitantly reversing inhibitor sensitivity. The induced insensitivity of the ATPase activity to DCCD, oligomycin and DBCT by oleate has been suggested

Table 5.5 Sensitivity of the ATPase activity of oleate treated submitochondrial particles to ATPase inhibitors

Addition	Specific ATPase activity µmol/min/mg protein	% Activity of control
None	2.00	100
1 mg serum albumin/mg SMP	1.94	97.0
Oligomycin (2 µg/mg protein)	0.07	3.5
Oligomycin + BSA	0.09	4.5
Oleic acid (500 nmol/mg)	3.20	160.0
Oleic acid + BSA	2.15	107.5
Oligomycin + oleic acid	3.30	165.0
Oligomycin + oleic acid + BSA	0.38	19.0
DCCD (5 nmol/mg protein)	0.04	2.0
DCCD + BSA	0.04	2.0
DCCD + oleic acid	3.25	162.5
DBCT (5 nmol/mg protein)	0.08	4.0
DBCT + oleic acid	3.40	170.0
Efrapeptin (2 µg/mg)	0.0	0.0
Efrapeptin + oleic acid	0.0	0.0

Submitochondrial particles suspended at 10 mg/ml were preincubated with various ATPase inhibitors for 30 minutes at 4° C. 2 mg aliquots of this suspension were preincubated with oleic acid (500 nmol/mg protein ± bovine serum albumin (1 mg BSA/mg SMP) at 25° C for 60 minutes. The BSA was added after 30 minutes preincubation with oleic acid. ATPase activity was then assayed as described in Methods. Each value presented in the table is the mean of 5 duplicates.

by Bertoli (239) to be due simply to the sequestering of the inhibitors by oleate, making them unavailable for inhibition. However, such a mechanism could not be used to explain the induced insensitivity to DCCD which, unlike DBCT and oligomycin is a covalent inhibitor of the ATPase (181).

The insensitivity of the ATPase of oleate-treated submitochondrial particles to DCCD has also been reported by M. Carver (240) who suggested that oleic acid was playing a cofactor role in the ATPase reaction. This, in my view was a gross mis-interpretation of the results.

5.5 CONCLUSION

The results presented in this Chapter have shown that most fatty acids and their acyl-esters are potent uncouplers of oxidative phosphorylation and energy-linked reactions at relatively low concentration (100 nmol/mg). At higher concentration, 200-500 nmol/mg, they stimulate ATPase activity and induce insensitivity to oligomycin, DCCD and DBCT in mitochondria and submitochondrial particles, which is reversible by treatment with serum albumin. The acyl-CoA esters at low concentration, (10-20 nmol/mg protein) inhibit ATP synthesis only in intact mitochondria, where they inhibits the ADP/ATP translocase. The results do not indicate a cofactor role for oleic acid or any of its acyl-ester derivatives in oxidative phosphorylation or energy-linked reactions.

CHAPTER 6

6. ENZYMIC METHOD FOR THE QUANTITATIVE DETERMINATION OF NANOMOLE AMOUNTS OF TOTAL LIPOIC ACID AND LIPOAMIDE

6.1 INTRODUCTION

α-Lipoic acid (1,2-dithiolane-3-valeric acid) was discovered independently in several laboratories in the late 1940's, and was shown to be an accessory growth factor for a variety of micro-organisms (201-207). It is a five-membered cyclic dithiolane with a five carbon carboxylic acid side chain (Fig. 6.1). The pure crystalline compound is pale yellow in colour (λ_{max} 330 nm), has a pKa of 4.7 and is optically active ($[\alpha]_D^{25}$ = 96.7). The chiral centre at C6 of the lipoic acid molecule is responsible for the optical activity. Lipoic acid is relatively stable in the solid state. However, when it is heated above its melting point, or its solutions are exposed to light, it undergoes thermal or photochemical fission of the disulphide linkage into thiyl radicals which subsequently polymerise (208). α-Lipoic acid is readily oxidised to the sulphoxide (β-lipoic acid) with mild oxidising agents such as hydroperoxides, or when allowed to stand in air (207). The unique reactivity and instability of lipoic acid has been attributed to the ring strain inherent in the

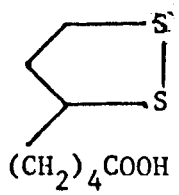


Fig. 6.1 Structure of lipoic acid

molecule (207, 209). The ring strain is thought to arise from electron repulsion (orbital overlap) between the filled non-bonding p-orbitals on adjacent sulphur atoms of the dithiolane ring. The ring strain has been estimated from spectral and thermal combustion analysis to have a value of 17-29 kJ/mole (207, 210). It is this high reactivity of the disulphide bond of the dithiolane ring that is responsible for the biological activity of lipoic acid.

Lipoic acid is widely distributed among micro-organisms, plants and animals. Although it has been shown to stimulate growth in micro-organisms (201-207), it has never been shown to stimulate growth response in higher animals. However, there is no doubt that lipoic acid plays a vital role in animal metabolism. At present, the only well defined role of lipoic acid is that of a prosthetic group in multienzyme complexes, which catalyse the oxidative decarboxylation of α-keto acids, such as, pyruvate and α-ketoglutarate (207, 211-214). The presently accepted mechanism of the pyruvate dehydrogenase complex, which catalyses the overall reaction :

$$CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}COOH + NAD^+ + CoASH \rightarrow CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}SCoA + CO_2 + NADH + H^+,$$

outlined in Fig. 6.2. The mechanism depicts a central role for lipoic acid, which serves a physical transport role within E_2 (Fig. 6.2) in addition to its chemical participation in the reaction sequence (212, 214). Recently, proposals implicating an energy coupling role for lipoic acid during photosynthetic and oxidative phosphorylation have been put forward by a number of workers (132). However, in studies carried out on spinach chloroplast (215), E. coli lipoic acid auxotrophs (216, 217) and beef heart mitochondria (see Chapter 4 of this thesis), no evidence was obtained to support these proposals.

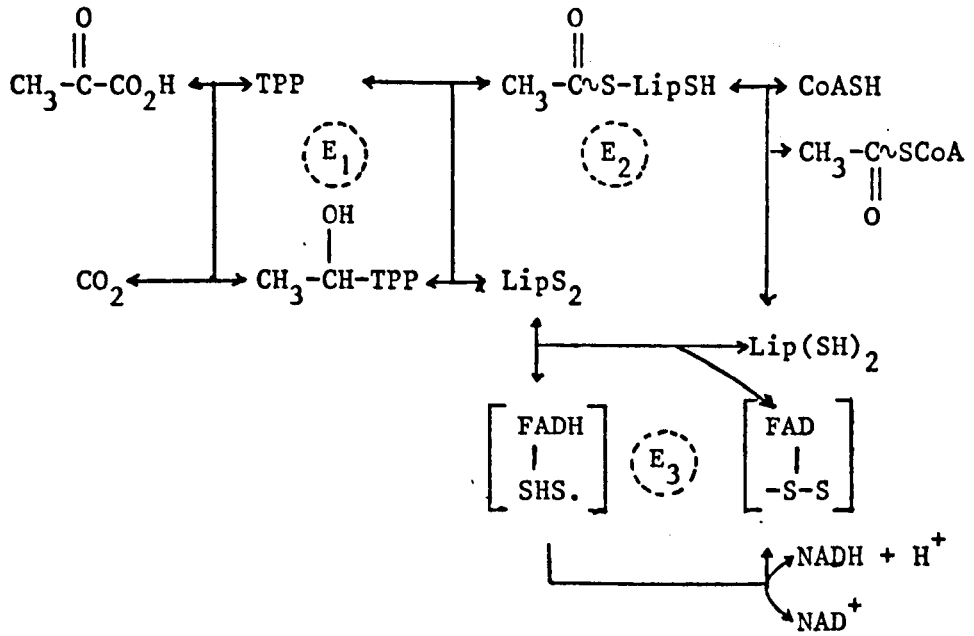


Fig. 6.2 Reaction mechanism of pyruvate dehydrogenase complex of E. coli. E_1, pyruvate decarboxylase; E_2, lipoyl-transferase; E_3, dihydrolipoic acid dehydrogenase; TPP, thiamine pyrophosphate (see refs. 207, 212-214).

The conservative cellular distribution of lipoic acid and its apparent involvement in a number of biological functions, have generated continual interest for methods of analysis of this cellular component. Several turbidimetric and monometric methods [see (218) for review] have been described for the assay of lipoic acid. Most of these methods utilise the growth requirement of certain micro-organisms for lipoic acid. More recently, mutants of E. coli that are unable to synthesise lipoic acid have been characterised and found to have a lipoic acid requirement of 0.5 ng/ml for half maximal aerobic growth (219). These bioassay techniques are very sensitive; lipoic acid at levels of 0.2-2.0 ng and 5-50 ng can be measured with the turbidimetric and polarographic methods respectively. However, the values obtained are usually very sporadic and unreliable. This is due to the dependence of growth of the micro-organisms on such variables as pH and temperature,

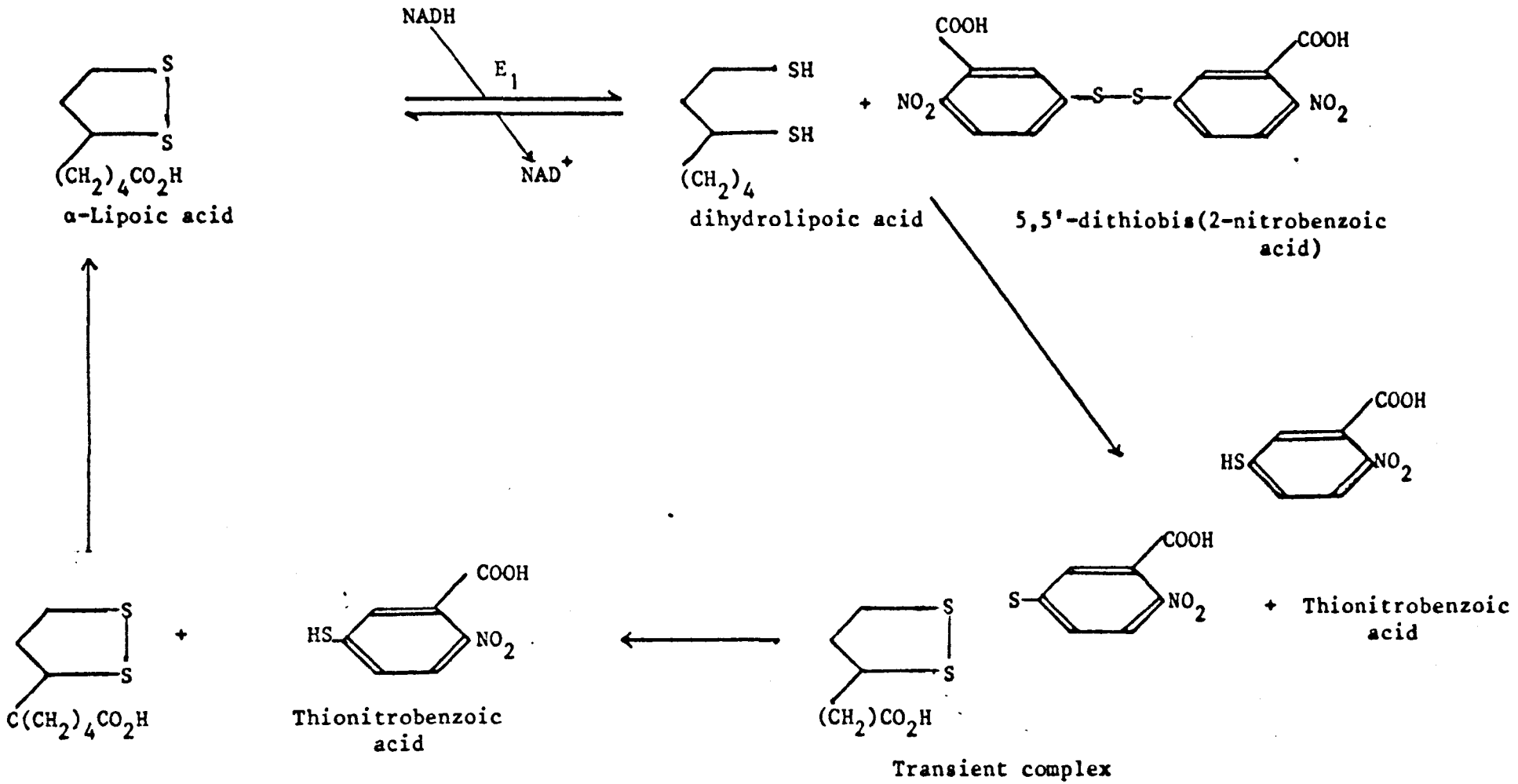


Fig. 6.3 Proposed mechanism for the catalytic reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to thionitrobenzoic acid by NADH in the standard assay system. E_1 is lipoamide dehydrogenase.

fluctuation of which produces erratic growth. Furthermore, these methods of analysis are very laborious and can take up to six days to assay a single sample (218).

In this Chapter we report in detail, the composition, mechanism and characteristic properties of a method for the measurements of nanomole amounts of lipoic acid. The method is based on the reduction of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) by NADH, in the presence of catalytic amounts of lipoic acid (or lipoamide), in a system in which dihydrolipoic acid is continually regenerated from its oxidised form, in a cyclic manner. The mechanism on which this method is based is outlined in Fig. 6.3 and is essentially the same as that proposed by Perham et al. (214) for the reduction of DTNB by the α-keto acid dehydrogenase complexes. The mechanism depicts a coupled assay system in which the formation of dihydrolipoic acid from oxidised lipoic acid in the presence of NADH and lipoamide dehydrogenase, is followed by the rapid reaction of dihydrolipoic acid with DTNB to produce oxidised lipoic acid and 2-nitro-thiobenzoic acid. The 2-nitrobenzoate anion is a chromophoric ion, having a molar absorption coefficient of 13,600 at 412 nm (214) which is about twice that of NADH at 340 nm. The reaction can therefore be conveniently followed by monitoring the increasing absorbance at 412 nm. Unlike previous methods of analysis, the method described here measures the total lipoic (reduced + oxidised) content of unknown mixtures. It is not subjected to any appreciable interference by the presence of other thiol components.

6.2 MATERIALS AND METHODS

Reduced pyridine nucleotides, glutathione (reduced and oxidised) were obtained from Calbiochem. Cysteine hydrochloride,

pig heart lipoamide dehydrogenase were purchased from Sigma Chemical Company. DTNB was purchased from Aldrich Chemical Company. Lipoic acid, lipoamide and dihydrolipoic acid, 2,3-dimercaptopropanol, dithiothreitol and mercaptoethanol were obtained as indicated in Materials section of Chapter 2. Mitochondria and submitochondrial particles were prepared from beef heart as described in the Methods section of Chapter 2. Rat kidney, heart and liver homogenates were prepared by grinding the blotted and weighed tissues at 0° C in a Potter-Elvehjem apparatus with Teflon pestle. Pre-coated TLC plates were obtained as described in the Materials section of Chapter 3.

Hydrolysis and extraction of lipoate from tissues

Mitochondria (1.0 g), submitochondrial particles (0.4 g) and the 'total tissue homogenates' of liver, heart and kidney derived from rats were suspended at $\sim$ 20 mg/ml in 50 mM K H_2PO_4, 1 mM EDTA, pH 6.3 buffer. 4 ml aliquots of suspensions were placed in 5 ml long necked glass vials, and made $\sim$ 0.125 M NaOH by addition of the appropriate (100 µl) amount of 5 M NaOH each of which was sealed under an atmosphere of nitrogen. The vials were then autoclaved at 15 psi for 3 hours. The hydrolysates from each tissue were combined separately, diluted 1:1 with 3 x distilled water, acidified with 6 M HCl to pH 2-3, and extracted with 2 volumes of chloroform. Using a separating funnel, the lower organic layer containing the lipoic acid was run off into a round-bottomed flask and the remaining aqueous layer re-extracted with 2 vol. chloroform. The pooled organic extracts were rotary evaporated to remove the chloroform, and the residue resuspended in 0.5 ml of

chloroform. Samples of these extracts were 'spotted' onto analytical TLC plates and spread onto preparative TLC plates which had been previously cleaned by allowing the developing solvent to run to the top of the plates.

All the plates were developed in a chromatography tank containing 150 ml of chloroform/methanol (2:1), which had previously been allowed to equilibriate for 3 hours. The plates were visualised by exposure to iodine in an enclosed chamber, and the band(s) running in the position corresponding to authentic lipoic acid scraped off the preparative TLC plates. The scrapings were added to a round bottomed flask containing 50 mls of distilled chloroform and allowed to stand for 24 hours. The supernatant was decanted off into another round bottomed flask, rotary evaporated to dryness and the residue resuspended in 0.5 ml methanol. This solution was then assayed for lipoate in the standard assay system. Controlled samples containing known amounts of lipoate, in which the lipoate was protected by BSA (20 mg/ml) were treated similarly.

6.3 RESULTS

A preliminary investigation of the dependence of 5,5-dithiobis(2,nitrobenzoic acid) (DTNB) reduction on the components of the assay system, resulted in the adaptation of the experimental arrangement shown in Table 6.1, for the routine assay of lipoic acid. This protocol, which is referred to hereafter as the 'standard assay system', makes use of two reaction mixtures balanced with respect to all components except lipoic acid or lipoamide. The employment of such a balanced system was dictated by the necessity for correction of the background rate of DTNB reduction by the

NADH, lipoamide dehydrogenase pair alone (see Fig. 6.4, line 1), a reaction which becomes significant at low concentrations of lipoate. The employment of the balanced system coupled with the use of a double beam spectrophotometer, allows automatic corrections to be carried out.

Table 6.1 Standard assay system

Test cuvette	Amount	Blank cuvette
50 mM phosphate buffer (pH 6.3)	1.0	50 mM phosphate buffer
DTNB	0.6 µmole	DTNB
Lipoamide dehydrogenase	100 µg	Lipoamide dehydrogenase
Lipoic acid or lipoamide	x nmoles	-
NADH	1.2 µmol	NADH

Components were dissolved in 50 mM sodium phosphate/0.5 mM EDTA buffer pH 6.3, and were added in the amounts and in the order indicated. Lipoic acid or lipoamide were added as methanolic solutions. The final volume was 1.36 ml. The rate of reaction at 20° C was usually expressed as the change in absorbance per 6 minutes at 412 nm. The reaction was initiated by the addition of NADH.

Typical photometric tracings resulting from the reduction of DTNB in reaction mixtures containing 0-50 nmol lipoic acid and 0-4 nmol lipoamide are shown in Fig. 6.4. Fig. 6.4 shows that the rate of reduction of DTNB (i.e. the rate of increase of absorbance at λ_{max} 412 nm) on addition of NADH to initiate the reaction, is linear with time, well beyond the 6 minutes employed as the basis of measurement. The results presented on Fig. 6.5 show that the rate of DTNB reduction is independent of NADH concentration above 200 µM. This may be due to the fact that at 200 µM, the concentration of the NADH is 8-10 fold greater than the K_m (20-30 µM) of the enzyme for NADH

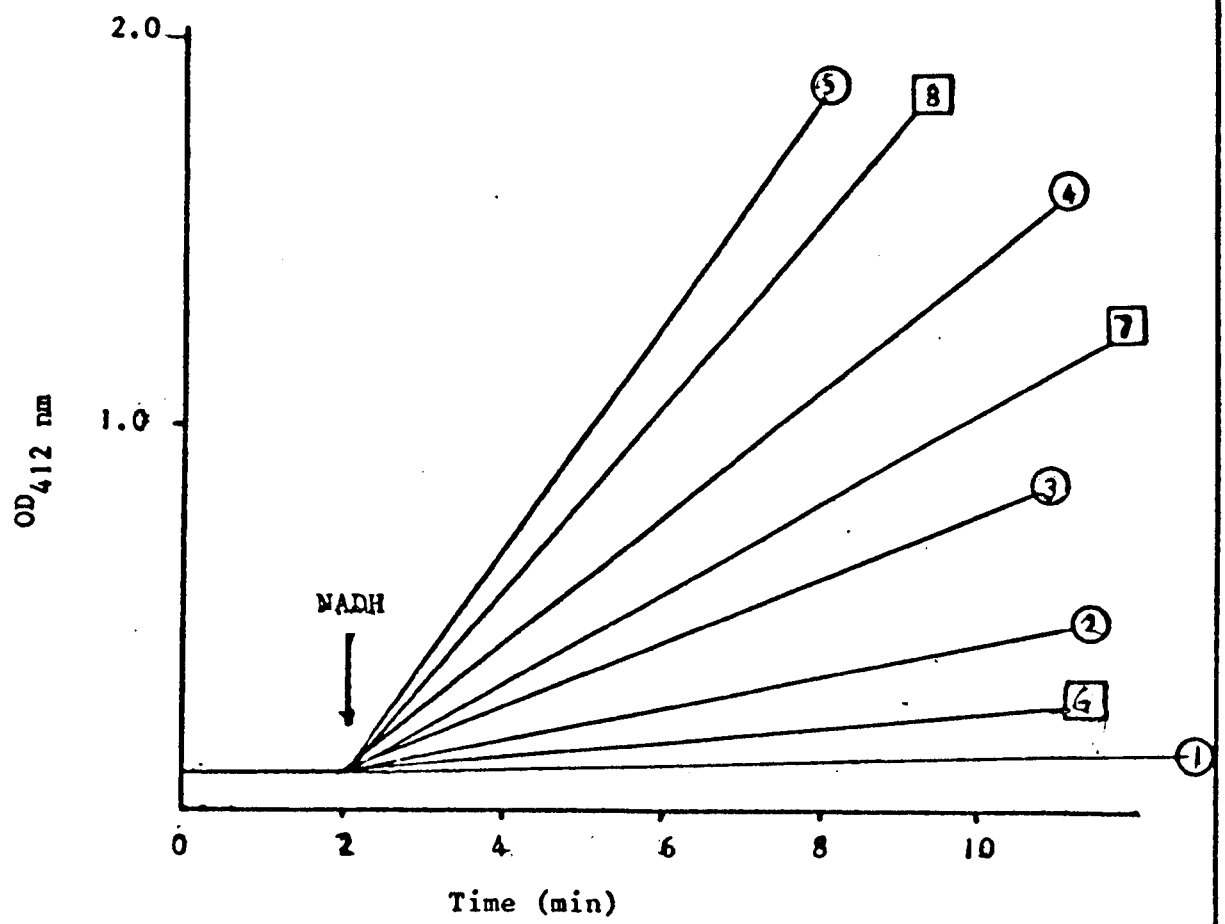


Fig. 6.4 . Typical spectrophotometric tracings obtained during reduction of DTNB by catalytic quantities of lipoic acid or lipoamide in the standard assay system containing NADH pig heart lipoamide dehydrogenase

Tracings were produced with the balanced system of Table 6.1 using (1), 0.0; (2), 10; (3), 20.0; (4), 50.0; (5), 75.0 nmol lipoic acid and (6), 0.2; (7), 2.0; (8), 4.0 nmol lipoamide. The system containing all the components except NADH was incubated for 2 minutes. NADH was then added to initiate the reaction.

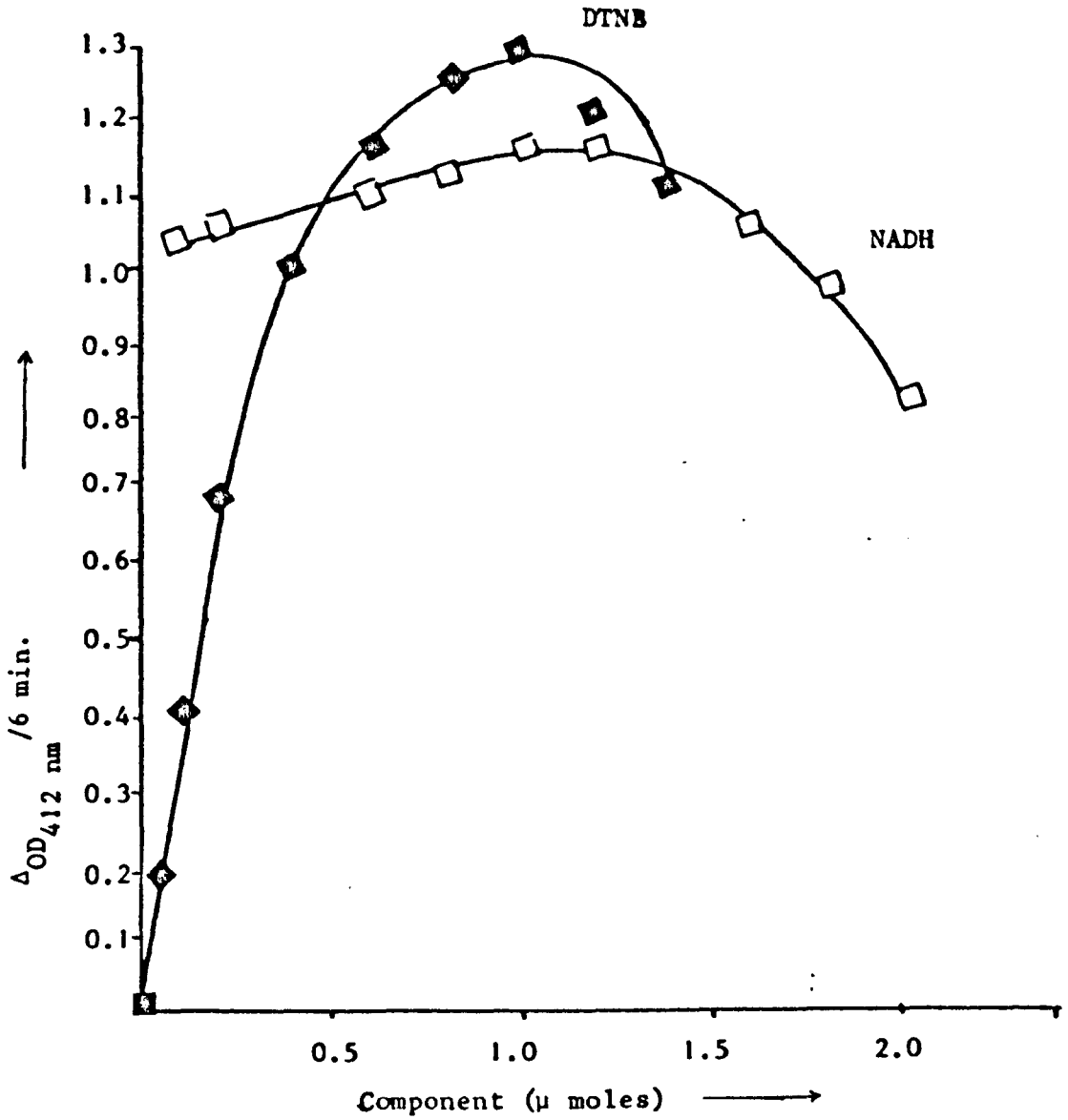


Fig. 6.5 Dependence of the rate of DTNB reduction in the presence of catalytic amounts of lipoic acid on the concentration of the components of the 'standard assay system'.

50 nmol lipoic acid was added to the assay system containing 100 μg lipoamide dehydrogenase, 1.0 μM NADH and the DTNB concentration varied or 50 nmol lipoic acid, 100 μg lipoamide dehydrogenase and 0.5 μmol DTNB and the NADH concentration varied. The data is presented on the figure as the $\Delta OD_{412\ nm}$/6 min against the concentration of the varied component. □ , NADH; ◘ , DTNB.

(207, 210). Like NADH, DTNB at concentrations above that giving maximal reaction rate (0.6 mM) cause inhibition of the rate of DTNB reduction (Fig. 6.5). This inhibition may be due in part to the susceptibility of the lipoamide dehydrogenase to inhibition by sulphydryl reagents in general, or to diminution in the amount of lipoic acid formed cyclically in the reaction. The rate of the reaction was found to be proportional to the amount of enzyme added to the system. Systematic experiments showed that the rate of reduction of DTNB in the presence of catalytic amounts of lipoic acid or lipoamide, was not influenced by the order of addition of the components listed in Table 6.1. However, it was considered desirable to follow the specific order indicated in the foregoing protocol. This allows sufficient time for the nonenzymic reaction of DTNB with thiol components (e.g. cysteine) present in the mixture undergoing analysis, prior to initiation of the enzymic reaction.

Fig. 6.6 and 6.7 show that the rate of DTNB reduction ($\Delta OD_{412\ nm}$ /6 minutes) is directly proportional to the concentration of lipoic acid or lipoamide added to the system. Lipoamide was also found to be 20 fold more effective as a catalyst than lipoic acid in the reduction of DTNB. This difference in the rate of DTNB reduction, catalysed by lipoamide and lipoic acid is a direct 'reflection' of the difference in the rates of reduction of lipoamide and lipoic acid by the lipoamide dehydrogenase. The enzyme has a greater specificity for lipoamide than for lipoic acid and has been shown to reduce lipoamide 20 times faster than lipoic acid (207, 210). The fact that the enzyme has a greater specificity for lipoamide than for lipoic acid, means that smaller quantities of lipoamide than lipoic acid can be determined. Thus, while it was possible to measure 0.1 nmol lipoamide fairly accurately, the limiting value of lipoic acid was approximately

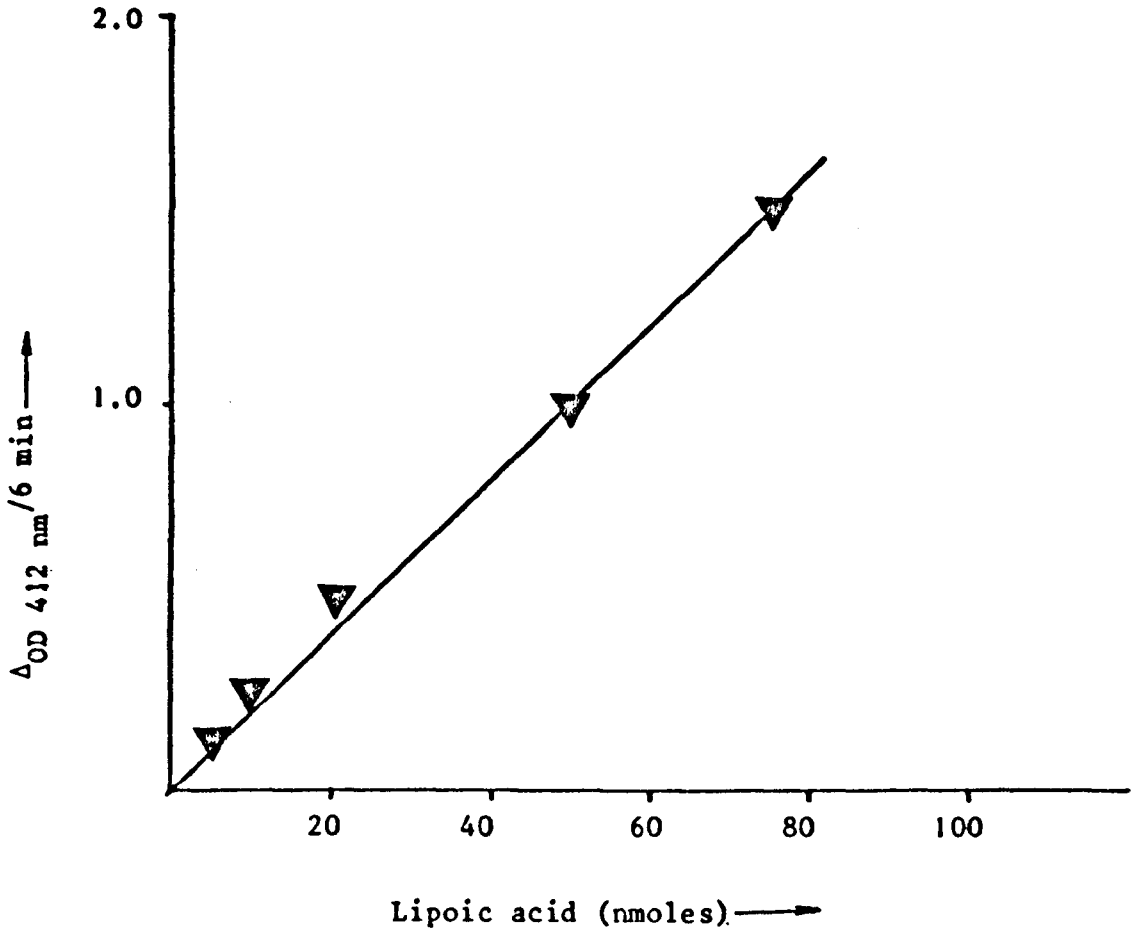


Fig. 6.6 Calibration curve for lipoic acid

DTNB reduction was assayed as described in Table 6.1. Each data point on the graph is the mean ± s.d. of 5 duplicates.

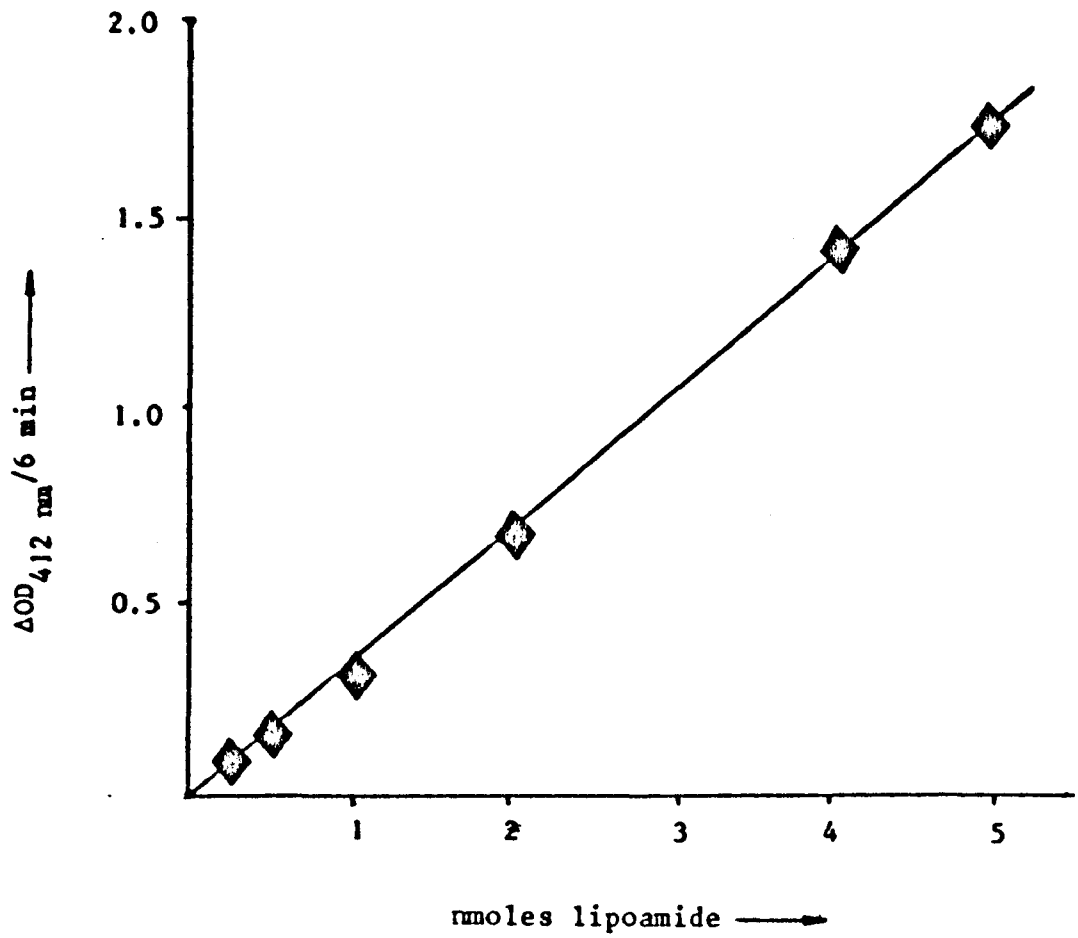


Fig. 6.7 Calibration curve for lipoamide

DTNB reduction was assayed as described in Table 6.1. Each data point on the graph is the mean ± s.d. of 4 duplicates.

1.0 nmol under the conditions of the standard assay system. Although, under slightly modified conditions (time period > 6 minutes) and with the use of scale expansion it is possible to extend the method to measure smaller values; under the latter conditions the background reduction of DTNB would become very significant at such low levels of lipoate, requiring considerable care to be taken in the preparation of the respective reaction mixutres. It is nevertheless evident from Figs. 6.6 and 6.7 that the rate of DTNB reduction ($\Delta OD_{412\ nm}$/6 minutes) maintains the same rectilinear dependence on lipoate concentration at the lower concentrations as would be expected from data obtained within the higher range of concentration.

In order to obtain some indication of the specificity of the enzymic assay for lipoic acid and lipoamide, systematic experiments were carried out in the presence of catalytic amounts of reduced glutathione (GSH); oxidised glutathione (GSSG); cysteine (CySH); 2,3-dimercaptopropanol, mercaptoethanol and dithiothreitol. The results presented in Table 6.2 show that the addition of any of the above mentioned thiol or dithiol to the 'standard assay system', had no effect on the rate of DTNB reduction in the presence of lipoic acid (or lipoamide). Fig. 6.8 represents a typical spectrophotometric tracing obtained, when a monothiol such as glutathione or a dithiol such as 2,3-dimercaptopropanol is added to the standard assay system. Fig. 6.8 shows that, after an initial increase in optical density at 412 nm due to the reaction of DTNB with the added free thiol groups, there is no further increase on addition of NADH to the system containing no lipoate (curve 1). However, on addition of lipoate before (curve 3) (or after) the addition of NADH, the reduction of DTNB proceeded at the normal rate (curve 1). The results presented in Table 6.2 and on Fig. 6.8 confirm the impression that the enzymic

Table 6.2 The effect of monothiols and dithiols on the rate of DTNB reduction (i.e. $\Delta OD_{412\ nm}$/6 minutes)

Additions		$\Delta OD_{412\ nm}$/6 minutes
Lipoate	Thiol/Dithiol	
Lipoic acid (50 nmoles)	-	1.06 ± 0.02
"	Cysteine	1.0 ± 0.04
"	Glutathione (reduced)	1.04 ± 0.0
"	Glutathione (oxidised)	1.02 ± 0.0
"	2,3-dimercaptopropanol	1.08 ± 0.06
"	Dithiothreitol	1.02 ± 0.02
Lipoamide (5 nmoles)	-	1.74 ± 0.08
"	Cysteine	1.76 ± 0.02
"	Glutathione (reduced)	1.72 ± 0.0
"	Glutathione (oxidised)	1.70 ± 0.02
"	2,3-dimercaptopropanol	1.68 ± 0.06
"	Dithiothreitol	1.74 ± 0.04

DTNB reduction was assayed as described in Table 6.1, 50 nmole lipoic acid or 5 nmoles lipoamide was used as catalyst as depicted above. In all cases 20 nmoles of the thiol or dithiol was added to the standard assay system prior to initiation (t = 0) of the reaction with NADH. The values are the mean ± s.d. of 4 duplicates.

procedure described here constitutes a sensitive and specific method for the determination of the total lipoic acid (and/or lipoamide) content of unknown mixtures.

In order to evaluate the usefulness of the foregoing analytical procedure, the lipoic acid content of a limited number of mammalian tissues were determined. The results presented in Table 6.3 indicate that the lipoate content of the tissues investigated

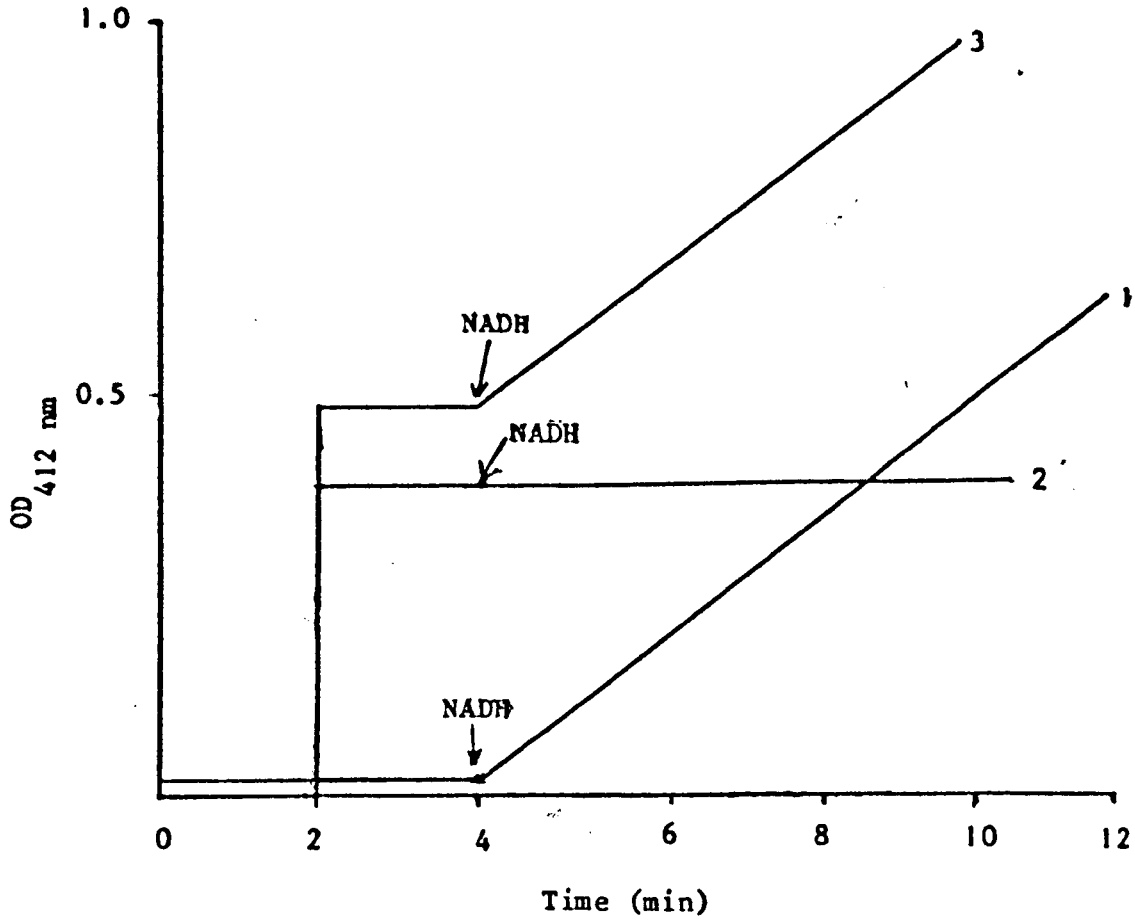


Fig. 6.8 The effect of 2,3-dimercaptopropanol on lipoic acid catalysed reduction of DTNB

DTNB reduction was assayed as described in Table 6.1. 20 nmoles 2,3-dimercaptopropanol was added to standard assay system containing 0.0 nmoles (curve 2) 20.0 nmol (curve 3) lipoic acid. The system was incubated for 2 minutes and NADH added to initiate the reaction. Curve 1 is the control system containing only 20 nmole lipoic acid.

Table 6.3 Lipoic acid content of selected mammalian tissues and organelle

Tissue	Lipoic acid content pmol/mg protein
Rat liver	41.2 ± 4.2
Rat kidney	8.6 ± 4.2
Rat heart	15.8 ± 3.8
Ox heart mitochondria	170.0 ± 12.5
Ox heart submitochondrial particles	65.0 ± 8.6

The lipoic acid content of the methanolic solution of the purified (final) extract (see Method) was assayed for lipoic acid in the standard assay system, as described in Table 6.1. The data is expressed in the table as picomol/mg protein. The data values represent the mean ± s.d. of 10 duplicates, in the case of liver, 4 duplicates, mitochondria; 4 duplicates, submitochondrial particles; and 4 duplicates of rat kidney and heart, in which the heart and kidney of 5 rats were pooled.

was very low, ranging from ∿ 9.0 pmol/mg protein in kidney to 41.2 pmol/mg protein in liver. Mitochondria, containing 170 pmol/mg protein was found to have the highest lipoate content. This seems to suggest that lipoate is concentrated in the mitochondria, possibly in 'bound' form. The fact that some lipoic acid is found in preparations of submitochondrial particles (65 pmol/mg protein) could be due to entrapment of lipoic acid and/or the presence of bound lipoic acid on the submitochondrial particles.

6.4 DISCUSSION

In considering the underlying mechanism of the standard assay, evidence is provided which proves that the catalytic action of lipoate residues in its continual regeneration as outlined in

Fig. 6.3. For example, under the conditions of the experiment, in the absence of a cycling process, the maximal optical density that would be obtained with 20 nmol dihydrolipoic acid (40 nmol thiol) at 412 nm is 0.4. The fact that the optical density is more than twice this value after 10 minutes (> 2.0) and increaseswith time (Fig. 6.4), indicates that cyclisation of the lipoic acid is occurring. Further evidence in support of a cyclisation process, comes from the finding that cyclisation occurs when dihydrolipoic acid is added to the assay instead of oxidised lipoic acid (Fig. 6.9). Cyclisation to form the oxidised lipoic acid from the mixed disulphide form carrying one thiobenzoate group, has been demonstrated by Brown et al. (214) with free dihydrolipoamide in solution. Results of experiments in which limiting amounts of DTNB was added to the standard assay system containing a catalytic amount of lipoic acid (50 nmol) is shown in Fig. 6.10. The results show that complete reduction of the added DTNB (15 nmol) occurs, and that while the rate of DTNB reduction depended on the concentration of lipoic acid, the end point of the reaction depended only on the amount of DTNB present in the system.

DTNB is catalytically reduced in the presence of NADH by 2-oxoglutarate dehydrogenase complex of beef heart (220), pyruvate dehydrogenase complex of yeast and their respective lipoyl transacetylase-lipoamide dehydrogenase sub-complexes, but not by the free lipoamide dehydrogenase component in the absence of free lipoate (214, 220). To explain these reactions Erfle and Saucer (220) advanced a mechanism in which the lipoamide dehydrogenase component of the complex catalytically reduced a mixed disulphide formed by reaction of one molecule of dihydrolipoic acid with two molecules of DTNB. However, in agreement with Brown et al. (214) we believe that the mechanism

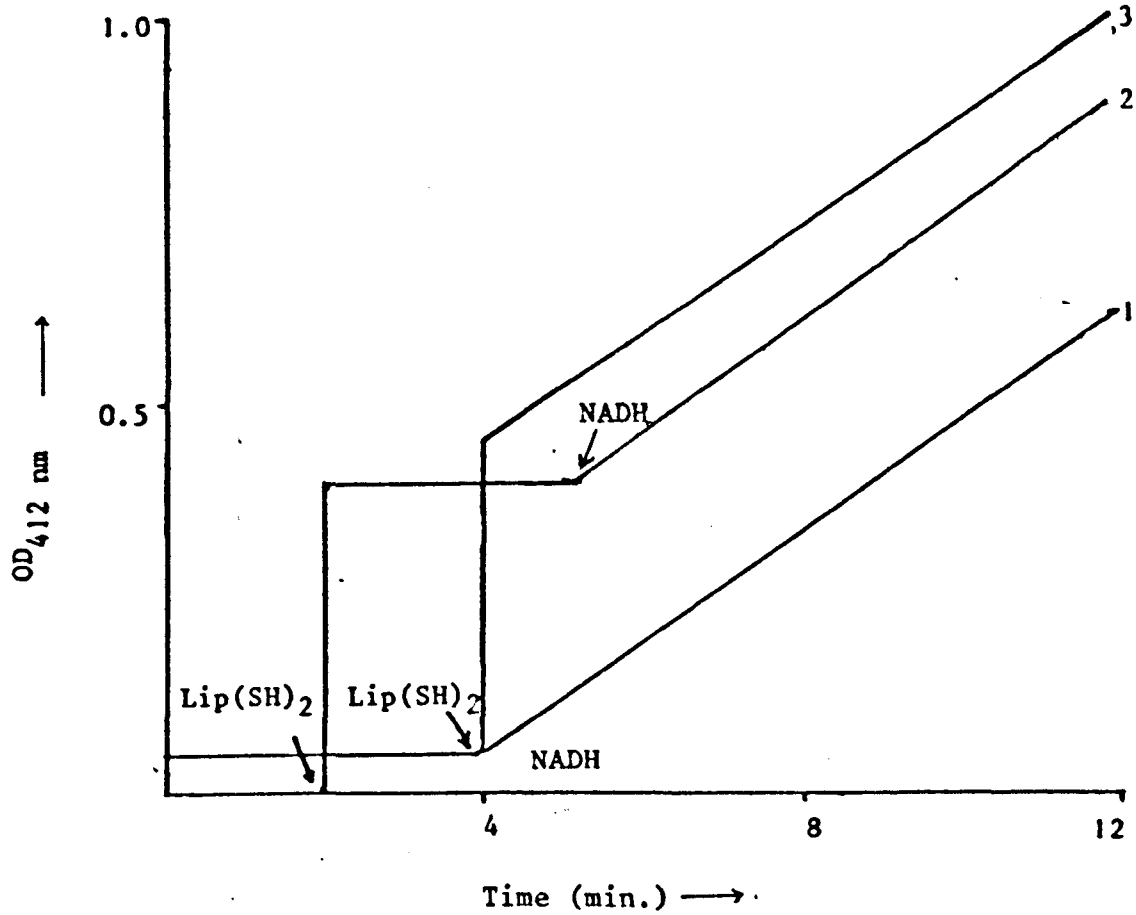


Fig. 6.9 Reduction of DTNB in the standard assay system with dihydrolipoic acid as a catalyst

The standard assay system containing 20 nmol lipoic acid (curve 1) or 20 nmol dihydrolipoic acid (curve 2) was preincubated for 4 minutes and the reaction initiated with NADH. In curve 3 (20 nmol)dihydrolipoic acid was used to initiate the reaction.

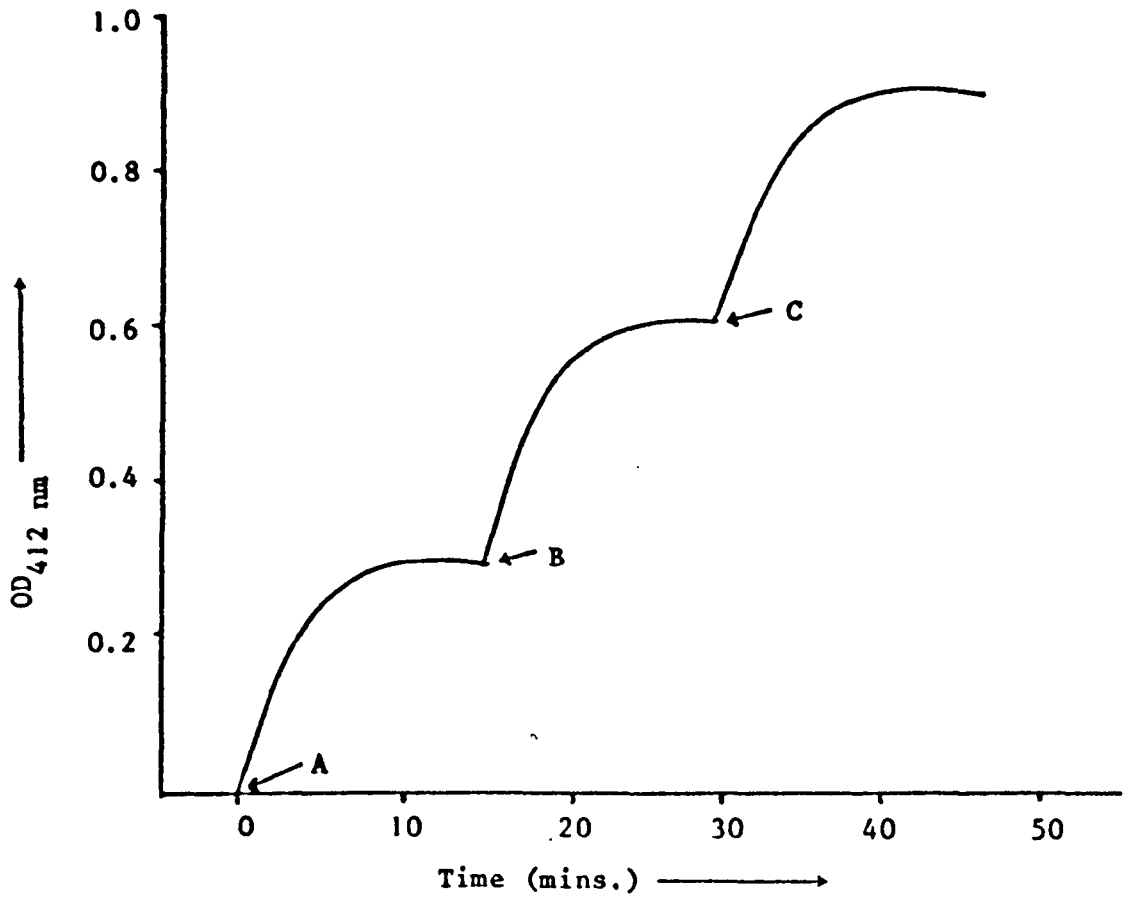


Fig. 6.10 Reduction of limiting amounts of DTNB in the standard assay system

DTNB reduction was assayed as described in Table 6.1. Small aliquots (15 nmoles in 5 µl) of DTNB were added to the reaction mixture containing (50 nmol) lipoic acid and NADH. DTNB was used to initiate the reaction. After complete reduction of the added DTNB occurred, further aliquots were added as indicated by the arrows (at B and C).

depicted in Fig. 6.3 is more likely than that proposed by Erfle and Saucer, for several reasons. Firstly, unlike the mechanism of Erfle and Saucer, it does not require the lipoamide dehydrogenase to catalyse a unique reaction. Secondly, the cyclisation to reform the oxidised lipoic acid from the mixed disulphide form carrying one thionitrobenzoate (see Fig. 6.3) can be observed with free dihydrolipoic acid or dihydrolipoamide (214) in solution, showing that this is a purely chemical step, which occurs spontaneously. Thirdly, it was found by titration that one mole of DTNB reacted with one mole of dihydrolipoic acid to give 2 moles of thionitrobenzoic acid. Finally, Wassarman and Major (221) have shown that DTNB catalyses the formation of an intrachain disulphide bridge between two cysteine residues in the active site of glyceraldehyde-3-phosphate dehydrogenase, by a similar mechanism.

The utility of the 'standard assay' has been explored by conducting a number of lipoate assays on selected mammalian tissues and organelle. Although no comparable data to those presented in Table 6.3 has been reported for the lipoate content of beef heart mitochondria and the heart and kidney of rats, the lipoate content of rat liver has been determined by various workers (218). A comparison of the lipoate content of rat liver determined by different assay procedures is presented in Table 6.4. The data shows that the values obtained were in the same order of magnitude, that is, pmoles/mg protein. However, the value obtained with the 'standard assay system' (41.2 $\pm$ 5.0 pmol/mg protein) was much higher than that obtained with the 'best' biological method (15.0 pmol/mg protein). However, this is not to say that the 'standard assay system' is more sensitive than the turbidimetric method using C.bovis, the main difference might be due to the preliminary treatment of the tissue

Table 6.4 Comparison of the lipoate content of rat liver, determined by different assay procedures

Method	Basis of method	Lipoate content pmoles/mg protein	(Ref.)
T. pyriformis		n.d.	(218)
C. bovis	Turbidimetric	15.0	(218)
C. bovis	Pad plate	10.0	(218)
S. faecalis	Pad plate	10.0	(218)
S. faecalis	Pyruvate oxidation (O_2 uptake measured)	n.d.	(218)
'Standard assay system'	DTNB reduction	41.2 ± 4.2 1.68 ± 0.38	(A) (B)

A and B indicate different methods of liberating free lipoic acid from its bound form. A - alkaline hydrolysis in an atmosphere of nitrogen ($NaOH/N_2$); B - acid hydrolysis in air (6M HCl); n.d. ≡ not determined.

to liberate free lipoic acid from its protein bound form. Whereas Stokstad et al. (from whose work some of the data in Table 6.4 was obtained) used acid hydrolysis in air to liberate the protein bound lipoate; alkaline hydrolysis in an atmosphere of nitrogen was the procedure adopted throughout this experiment. The latter method has been shown by Wagner et al. (222) and Beauclerk (223) to be the best method for liberating protein bound lipoate. These authors have shown that in test systems containing known amounts of lipoic acid, 80-90% of the added lipoic acid could be recovered using alkaline hydrolysis in an atmosphere of nitrogen compared with less than 5% under acid hydrolysis in air. The results presented in Table 6.4 show that under acid hydrolysis in air the measurable lipoate content of rat liver was 1.68 ± 0.38 pmol/mg protein. This value is less than 5% of the value obtained under alkaline hydrolysis in an atmosphere of nitrogen.

6.5 CONCLUSION

The 'standard assay system' provides a simple, reliable method for the determination of lipoic acid and lipoamide. Its advantages of speed, low cost and reproducibility make it a much more applicable method than all the biological methods reported (218). The only drawback of the enzymic system is that it cannot distinguish between lipoic acid and lipoamide.

The main stumbling block in measuring the lipoate content of mammalian tissue using the 'standard assay system' or any of the biological methods reported (218), is the preliminary treatment of the tissue to liberate and isolate the lipoate. Incomplete liberation of lipoate from its bound form (by hydrolysis) in addition to loss of lipoate due to its destruction during the hydrolytic and purification steps could lead to under-estimation of the true values.

APPENDIX I

Compounds that inhibit energy linked reactions

1. (a) F_1 ATPase inhibitors

   (b) F_1F_O ATPase inhibitors

2. Uncouplers

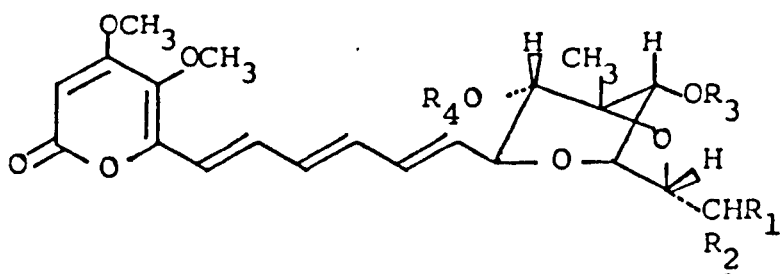
3. Ionophores

4. Electron transport inhibitors

1. ATPase inhibitors

(a) F_1ATPase inhibitors

Aurovertins.- The aurovertins are a class of compounds having the general formula:-



	R_1	R_2	R_3	R_4	molecular weight
Aurovertin A	H	Me	COMe	COMe	501
B	H	Me	COMe	H	445
C	H	H	COMe	H	451
D	OH	Me	COMe	H	475
E	H	Me	H	H	417

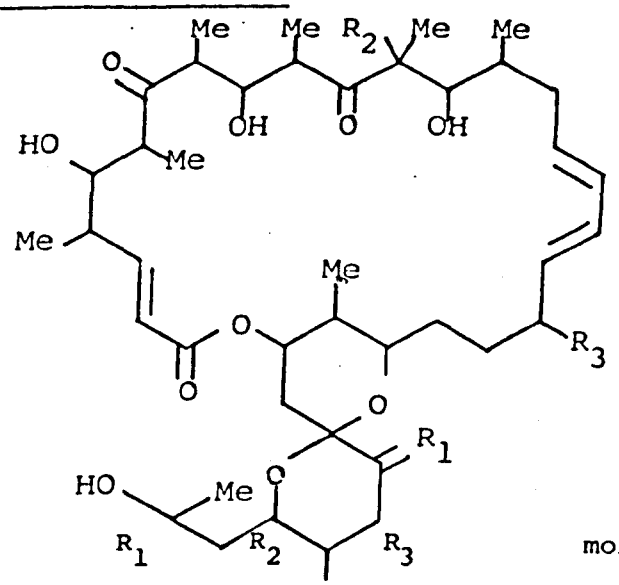
They are obtained from cultures of the fungus Calcorisporium arbuscula and are potent inhibitors of mitochondrial ATP synthesis; ATPase; $H_2{}^{18}O$-Pi exchange; ATP-^{32}Pi exchange; ATP-driven transhydrogenase; ATP-driven reversed electron transport; uncoupler induced swelling. D and B are the most potent forms.

The locus of action appears to be at the F_1 ATPase.

Efrapeptins.- Efrapeptins are a group of lipophilic polypeptides obtained from the hyphomycete Tolypocladium inflatum. The structures a unclear, but hydrolysis yields glycine, leucine and alanine in a ratio o 6:7:1 and an unknown amino acid. The polypeptides do not appear to possess N-terminal amino acids but do contain a free carboxyl group. They have a molecular weight somewhere in excess of 1500. They have a mode of action similar to that of the aurovertins but, although they act on the F_1 ATPase, they appear to have a binding site distinct from that of the aurovertins.

(b) F_1F_O ATPase inhibitors

Oligomycin



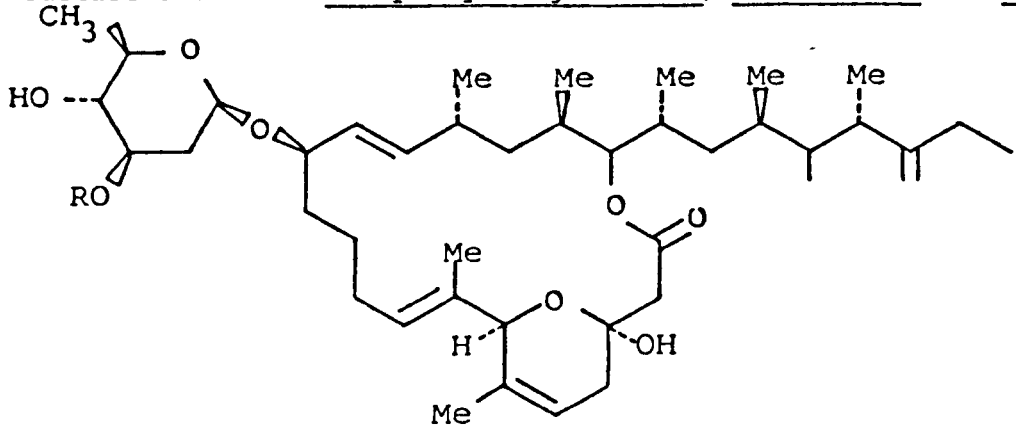
				molecular weight
Oligomycin A	H_2	OH	CH_3CH_2	760
B	O	OH	CH_3CH_2	784
C	H_2	H	CH_3CH_2	744
D	O	OH	CH_3	760

The oligomycins are a group of complex antibiotics obtained from cultures of Streptomyces diasto chromogenes (oligomycins A-C) and Streptomyces rutgersensis (NRRL. B-1256 (oligomycin D, also known as rutamycin)).

The oligomycins are the 'classical inhibitors' of the F_1F_O ATPase in mitochondria, and are among the most potent known: 1 molecule is thought to be sufficient to completely inhibit 1 molecule of ATPase.

It inhibits oxidative phosphorylation, ATP-Pi exchange, ATP-ADP exchange, F_1F_O ATPase and all energy linked activities consuming or producing ATP. It has no effect on the isolated F_1 ATPase. Its precise mode of action is unclear; it is thought either to block the formation of the '~P' state or to prevent proton pumping through the F_O portion of the ATPase. It probably interacts with 'subunit 9' of the ATPase. Sulphydryl groups have been implicated in its binding site.

Venturicidin The venturicidins are a group of compounds obtained from culture broths of Streptomyces griseolus, S. hastedii and S. xanthophaeu



Venturicidin A R = NH_2CO molecular weight 750

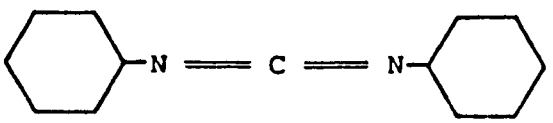
B R = H molecular weight 707

Venturicidin has a mode of action similar to that of the oligomycins although it is slightly less potent. It acts on the F_O portion of the ATPase, inhibiting all ATP utilising and producing reactions. It appears to bind to subunit 6 of the ATPase.

Leucinostatin This is a lipophilic polypeptide isolated from the fungu Penicillium lilacinum A-267. Hydrolysis yields leucine and 4 unknown amino acids. It has a complex mode of action, being an uncoupler in high concentration but appearing to act like oligomycin or venturicidin at lower concentrations. It has no effect on isolated F_1 ATPase.

Dicyclohexylcarbodiimide (DCCD) DCCD is a potent inhibitor of energy linked reactions. It appears to exert its inhibitory characteristics by covalently binding to subunit 9 of the F_O ATPase in mitochondria and

to similar low molecular weight proteolipids in chloroplasts and bacterial vesicles. It inhibits all ATP utilising and producing activities in an oligomycin-like manner. Its binding site appears to be separate from that of oligomycin, however.



DCCD

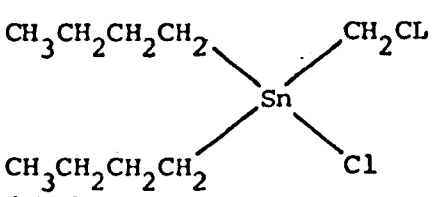
Trialkyltins These are compounds having the general formula

$R_1R_2R_3SnX$ where $R_1R_2R_3$ are hydrocarbon groupings

X = halogens or -OH (usually).

They are potent inhibitors of ATP utilising or producing energy linked reactions (the compounds where $R_1R_2R_3$ are: n-ethyl or n-butyl,n-propyl or phenyl appear to be the most effective). They do not inhibit F_1 ATPase activity and appear to bind to the subunit 9 component of the membrane bound (F_O) portion of the ATPase. They generally act like oligomycin but may also facilitate a $Cl^- \longleftrightarrow OH^-$ exchange reaction across the mitochondrial inner membrane.

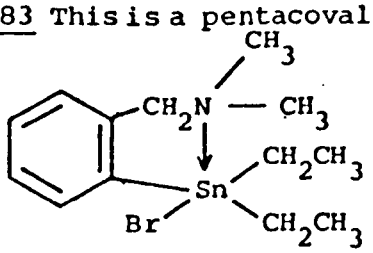
Dibutyl chloromethyl tin chloride This is a trialkyl tin, having the structure:-



molecular weight 318

It appears to bind to a nonprotein lipophilic component of the inner mitochondrial membrane. It has a similar efficacy, and general properties like, the other trialkyl tin compounds.

Compound VE2283 This is a pentacovalent organotin compound having the structur

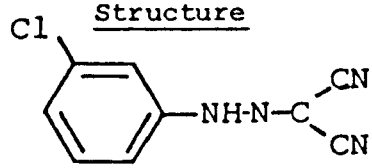
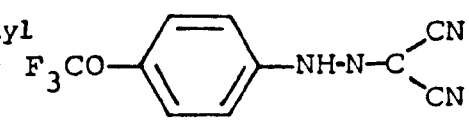
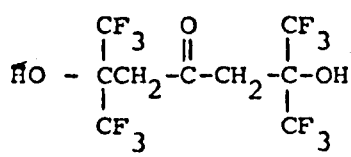
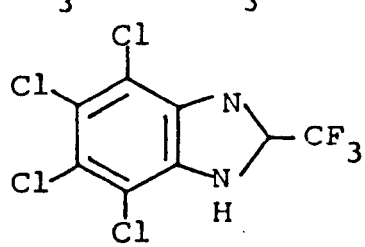
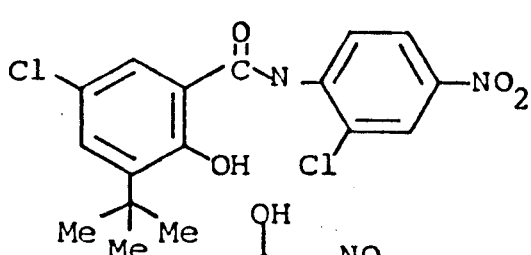
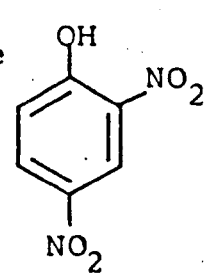
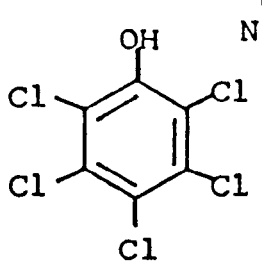


molecular weight 390

It appears to act like oligomycin and is at least as potent an inhibitor (on a molar basis). Its precise mode of action is at present under investigation.

2. Uncouplers

These are compounds that dissipate the high energy state, '$\sim$', of the inner membrane; whether they act by dissipating a proton gradient or in a more specific manner (e.g. binding to a component of the energy coupling apparatus) is not completely clear, although most are lipophilic weak acids or bases, capable of conducting protons across artificial lipid bilayers. For a more detailed discussion of the mechanism of uncoupling, see Chapter 2.

Name	Structure	Acronym
m-chloro carbonyl cyanide phenyl hydrazone		CCCP
trifluoromethoxy carbonyl cyanide phenylhydrazone		FCCP
bis hexafluoro acetonyl acetone		'1799'
4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole		TTFB
5-chloro-3-tert butyl-2'-chloro-4'nitrosalicylanilide		S-13
2,4-dinitrophenol		DNP
Pentachlorophenol		PCP

3. Ionophores

These are a group of compounds that have the ability to transport cations across lipid membranes. They uncouple oxidative phosphorylation either by collapsing the $\Delta\phi$ component of the proton motive force or by facilitating energy linked, futile cycles of ions across bioenergetic membranes, thus dissipating 'ν'.

Gramicidin D Isolated from cultures of Bacillus brevis. It is a lipophilic polypeptide consisting of 15 amino acids of alternating L and D forms, i.e.

HCO-val-gly-ala-leu-ala-val-val(tryp-leu)-tryp-$NHCH_2CH_2OH$

L L D L D L D

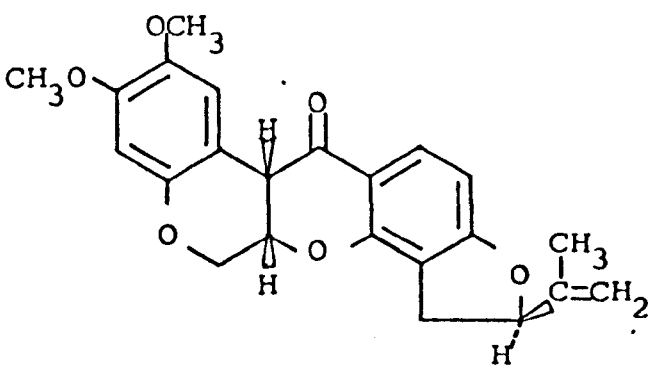
It is thought to adopt a helical form in the membrane, effectively poking a hydrophilic hole through it, through which ions can pass. It transport K^+ and H^+ with similar efficiencies (K^+ slightly > H^+).

Valinomycin This is isolated from Streptomyces fulvissimus. It is one of the 'cage' form ionophores which enclose cations with a hydrophobic shell. It contains 3 moles of L-valine, D-α-hydroxy isovaleric acid, D-valine, L-lactic acid, linked alternately to form a 36 membered ring. It principally transports potassium across membranes, but will also transport other monovalent cations with lowered effectivity.

4. Electron transport inhibitors

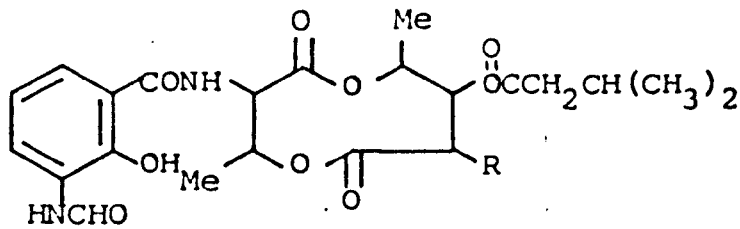
These are compounds that act by blocking electron flow between respiratory substrates and oxygen.

Rotenone Obtained from derris root. It appears to block electron transport between NADH dehydrogenase and ubiquinone.



molecular weight 393

Antimycin A Blocks electron transport between cyt b and cyt c; it binds to a protein subunit of complex III.



Antimycin A_1	R = n-hexyl	molecular weight 558
Antimycin A_3	R = n-butyl	molecular weight 530

Cyanide, azide, sulphide, carbon monoxide All block electron transport at the level of cytochrome oxidase. They act by irreversibly binding to the 'free ligand' of the iron in cytochrome a_3's haem grouping.

APPENDIX II

Lipoamide Dehydrogenase

This lipoamide dehydrogenase is present on 'well-washed' submitochondrial particles, prepared from beef heart mitochondria, as described in the Methods section of Chapter 2. The enzyme co-purifies with F_1-ATPase when the F_1-ATPase is prepared by chloroform extraction with the method of Tyler and Webb. However, the enzyme can be separated from the F_1-ATPase by ion-exchange chromatography on DEAE-Sephadex. Fig. $II_{(i)}$ shows the elution profile of the enzymes when the enzymic mixture bound on the column is eluted with buffers containing 20 mM tris-Cl, pH 8.0; 2 mM EDTA; 4 mM ATP and 0.1 M Na_2SO_4 or 0.15 M Na_2SO_4. Fig. $II_{(i)}$ shows that the F_1-ATPase which accounts for more than 90% of the total protein, is eluted off the column before the lipoamide reductase. SDS-gel electrophoresis not shown indicates an estimated molecular weight of the lipoamide dehydrogenase of 40-50,000.

The specific activity of the purified lipoamide dehydrogenase was 103 µmol/min/mg protein under optimum conditions (50 mM Pi, pH 6.5 2 mM lipoamide, 1.0 mM NADH). The reaction was followed by monitoring the oxidation of NADH (λ_{max} 340 nm). The pH profile of the 'membrane bound' enzyme and the purified enzyme were almost identical, and the optimum pH depended upon the direction of the reaction; that is on the initial substrates. For example, in the reduction of oxidised lipoic acid by NADH:

$$NADH + H^+ + LipS_2 \rightarrow NAD^+ + Lip(SH)_2$$

The optimum pH is 6.3-6.5, whereas in the reduction of NAD^+ by $Lip(SH)_2$ the reversed reaction of the optimum pH is 7.8-8.0.

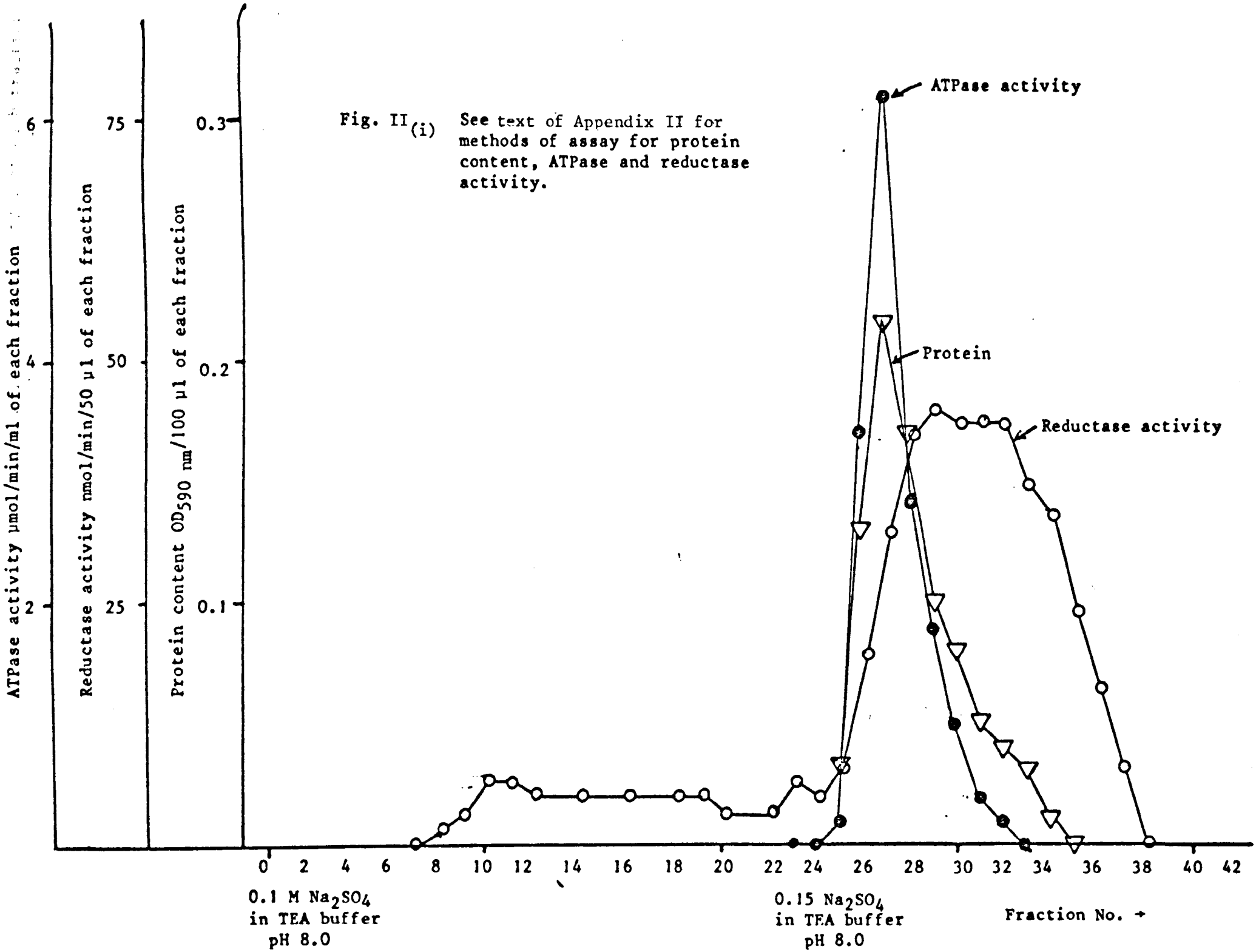


Fig. $II_{(i)}$ See text of Appendix II for methods of assay for protein content, ATPase and reductase activity.

Michaelis constants

	Substrate	Conditions
28.6×10^{-6} M	NADH	50 mM Pi, pH 6.5
1.86×10^{-3} M	Lipoamide	1 M EDTA

Enzyme and Protein Assays

ATPase activity was assayed as described previously in the Methods section of Chapter 2. Lipoamide reductase activity was measured by the method of Tasi, Templeton and Wand (256) 5-10 µg of the soluble enzyme preparation was added to a cuvette containing NADH (300 µM) and lipoamide (250 µM) in 50 mM phosphate buffer, pH 6.3; total 3.1 ml. The enzyme was used to initiate the reaction. The oxidation of NADH was monitored by following the decreasing absorbance at 340 nm in a SP 1800 spectrophotometer. When submitochondrial particles were assayed for reductase activity (50-100 µg) rotenone 1 µg/ml was added to the buffer.

Protein was assayed by the biuret method for (submitochondrial particles) and the folin method (for the soluble preparations) as described previously in Chapter 2.

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